THE EFFECT OF BIVALENT CATIONS ON SOME ENZYMES OF THE ALLANTOIN METABOLISM

CHR. VAN DER DRIFT

THE EFFECT OF BIVALENT CATIONS ON SOME ENZYMES OF THE ALLANTOIN METABOLISM

PROMOTOR: PROF. DR. H. BLOEMENDAL

Dit proefschrift werd bewerkt in het laboratorium voor Biochemie van de Katholieke Universiteit te Nijmegen onder leiding van Dr. Ir. G.D. Vogels.

2

THE EFFECT OF BIVALENT CATIONS ON SOME ENZYMES OF THE ALLANTOIN METABOLISM

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
DR. A.Th.L.M. MERTENS,
HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE,
VOLGENS BESLUIT VAN DE SENAAT
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 17 MEI 1968
DES NAMIDDAGS TE 4 UUR

DOOR

CHRISTIAAN VAN DER DRIFT

GEBOREN TE ARNHEM

1968
THOBEN OFFSET NIJMEGEN
DRUKKERIJ - UITGEVERIJ

Aan mijn moeder

Dr. J.M.F. Trijbels en Drs. J.C.M. Hafkenscheid ben ik dank verschuldigd voor hun medewerking aan de totstandkoming van dit proefschrift, alsmede de heer F.E. de Windt, die mij geassisteerd heeft tijdens het experimentele gedeelte van het onderzoek.
De tekeningen van dit proefschrift werden vervaardigd door de heer W.P.J. Maas van de Medische Tekenkamer (Hoofd: Chr. van Huijzen) en het fotografische gedeelte door de afdeling Medische Fotografie (Hoofd: A. Reijnen) van de Katholieke Universiteit te Nijmegen.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 ALLANTOIN AND ITS METABOLISM

1.1.1 *Distribution*

Allantoin is a product of purine catabolism. The substance is widely distributed in nature. It is excreted by vertebrates (fishes, amphibians, mammals) and invertebrates (crustaceans, ascidians, insects). It is found in several higher plants and presumably plays a role in transport and storage of nitrogen in these plants (MOTHES and ENGELBRECHT, 1953). A large group of microorganisms (algae, fungi, molds, bacteria) can degrade allantoin further.

An extensive survey of the literature about the occurrence of allantoin was given by VOGELS (1963) and recently by TRIJBELS (1967).

1.1.2 *Enzymes*

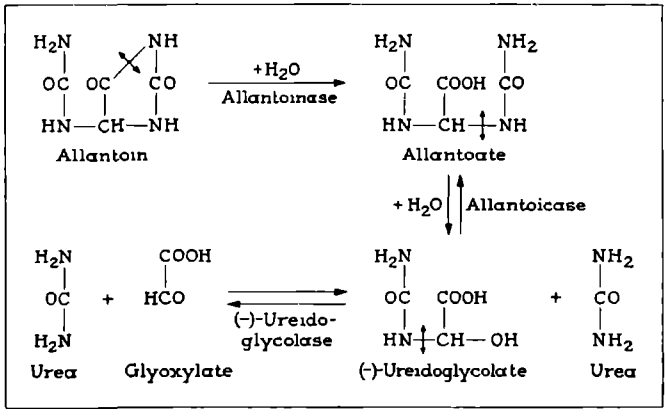
The metabolic conversion of uric acid *via* allantoin to glyoxylate and urea is called the uricolytic pathway. The enzymes involved in this pathway are: uricase (urate : oxygen oxidoreductase, EC 1.7.3.3), allantoinase (allantoin amidohydrolase, EC 3.5.2.5), allantoicase (allantoate amidinohydrolase), allantoate amidohydrolase and ureidoglycolase (ureidoglycolate amidinohydrolase). Reports on the occurrence and the properties of these enzymes, with references to the older and most recent literature, were given by VOGELS (1963), GUITTON, DURAND and BRUNEL (1965), FRANKE (1966) and TRIJBELS (1967).

At present there are known two complete routes of allantoin degradation. These are represented in Schemes 1 and 2, respectively. The first pathway appears to be the common route of allantoin degradation in animals, fungi and most bacteria *(e.g.* in *Pseudomonas aeruginosa;* TRIJBELS, 1967). The degradation route in higher plants is still un-

certain (VAN DER DRIFT and VOGELS, 1966). The second pathway is found in *Streptococcus allantoicus*, *Arthrobacter allantoicus* and in Escherichia species (VOGELS, 1963). The degradative pathway in *Pseudomonas acidovorans* and probably also in certain unidentified Pseudomonas species (BACHRACH, 1957) is given in Scheme 1 but allantoate is converted by allantoate amidohydrolase, the enzyme found only in the organisms which follow Scheme 2. Since *P. acidovorans* contained allantoate amidohydrolase activity (TRIJBELS and VOGELS, 1966a) it was postulated that ureidoglycine was an intermediate in this pathway. In contrast to the other bacteria which degraded allantoin *via* ureidoglycine this organism did not possess ureidoglycolate dehydrogenase activity (TRIJBELS, 1967).

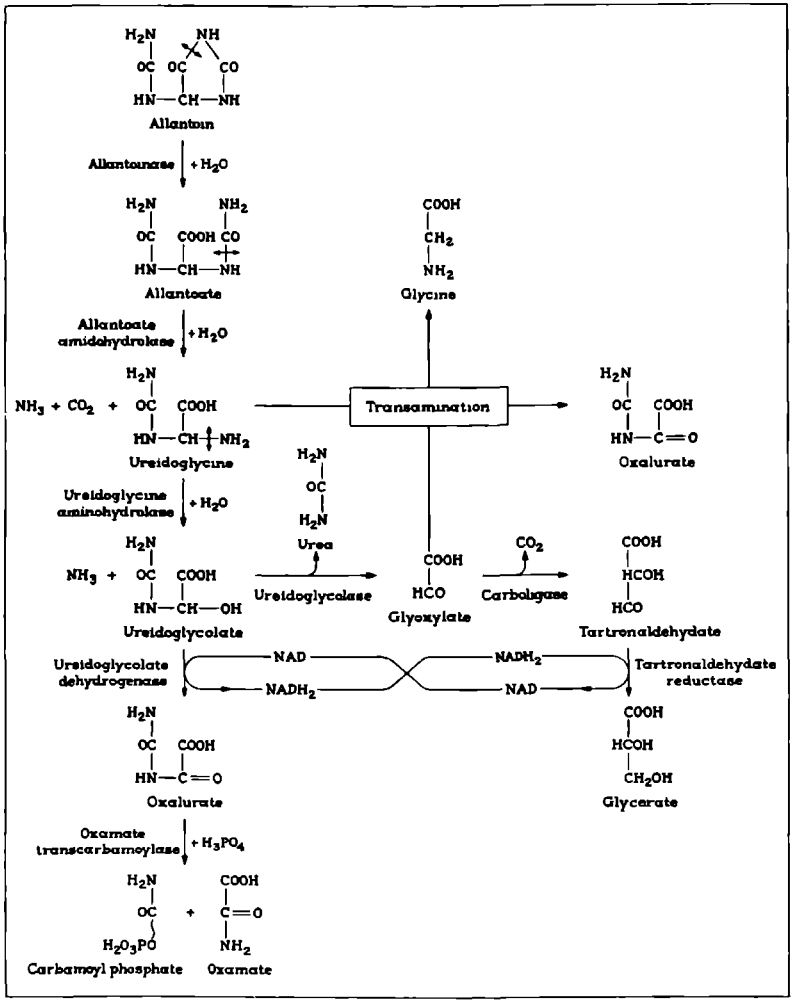
1.2 ROLE OF METAL IONS IN THE ALLANTOIN METABOLISM

Metal ions, especially Mn^{2+}, stimulate the reaction of many enzymes involved in allantoin degradation. Table 1 shows a summary of the stimulation by Mn^{2+} ions for several enzymes from the allantoin metabolism. Furthermore metal ions catalyze the non-enzymic hydrolytic cleavage of ureidoglycolate, an intermediate in allantoin degradation (TRIJBELS, 1967).



Scheme 1
Degradation of allantoate by *Pseudomonas aeruginosa* (TRIJBELS, 1967)

BRUNEL (1936) observed an induction of allantoinase in *Aspergillus niger* when Mn^{2+} ions were included in the growth medium, but VOGELS (1963) was not able to demonstrate such an effect with *A.allantoicus* after growth in a glucose-ammonium nitrate medium with or without $MnSO_4$ ($5x10^{-4}$M). Presumably the observed induction was due to co-factor activity of Mn^{2+} ions on the enzyme from *Aspergillus niger.*



Scheme 2

Degradation of allantoin by *Streptococcus allantoicus* and *Arthrobacter allantoicus* (VOGELS, 1963)

Table 1

Stimulation by Mn^{2+} ions of some enzymes involved in allantoin degradation

Organism	Allantoin-ase 1)	Allantoate amido-hydrolase 2)	Allantoic-ase 3)	Ureido-glycolase 2)	Oxamate trans-carba-moylase 2)
Streptococcus allantoicus	+	+	absent	+	+
Arthrobacter allantoicus	+	+	absent	+	+
Escherichia coli	+	+	absent		+
Pseudomonas aeruginosa		absent	+	-	absent
Glycine hispida L.	+				

1) VOGELS and VAN DER DRIFT, 1966; 2) VOGELS, 1963; 3) TRIJBELS, 1967.
+ = stimulation; - = no effect.

1.3 METAL-ENZYME INTERACTIONS

1.3.1 *Metalloenzymes and metal-enzyme complexes*

On the basis of interaction with metal ions the enzymes can be divided into two classes: 1) metalloenzymes and 2) metal-enzyme complexes (VALLEE, 1955, 1960; GURD and WILCOX, 1956; MALMSTRÖM and ROSENBERG, 1959).

To be classified as a member of the first group the enzyme must fulfill a number of characteristics (VALLEE, 1955): 1) a firm association between metal ion and protein must exist; consequently, the metal to protein ratio increases during purification and will remain constant when a homogeneous enzyme preparation is obtained; 2) an integral number of metal ions will be bound to a pure enzyme, indicating the

binding between distinct reactive ligands of the apoenzyme and the metal ion; 3) a stoichiometry must exist between the number of bound metal ions and prosthetic groups or coenzymes; 4) a correlation between the parameters protein, metal and specific activity is observed and on complete purification the ratio of metal content to specific activity will be constant; 5) the metal ion can only be removed from the enzyme with the aid of special techniques, with a concomitant decrease of catalytic activity; usually this process is reversible; 6) a small number of different metal ions is found up to now in metalloenzymes. Some examples are (Zn-)carboxypeptidase A (peptidyl-L-aminoacid hydrolase, EC 3.4.2.1; COLEMAN and VALLEE, 1961), (Fe, Mo-)xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2; AVIS, BERGEL and BRAY, 1956) and (Zn-)carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1; LINDSKOG and MALMSTRÖM, 1962).

A list of metalloenzymes was given by VALLEE and COLEMAN (1964). In some cases it was observed that the role of the metal ion in metalloenzymes only consisted in a maintenance of the quaternary structure of the protein (KÄGI and VALLEE, 1960).

In contrast to the first group in metal-enzyme complexes the metal ion is rather loosely bound to the protein part and dissociates readily, *e.g.* on dialysis. Diminution of the specific activity by removal of the metal ion can, like with the first group, be reversed by addition of the same metal ion or even another one, since the specificity with this kind of metal complexes is not as high as with metalloenzymes. The stoichiometry of association between metal ion and apoenzyme is not strictly defined and the amount of metal bound is largely determined by the concentration of free metal ions. A strict proportionality between metal content and specific activity is not observed. Metal ions influencing the reaction rate of this kind of enzymes are found in almost every group and period of the periodic system.

These two classes of metal-enzyme systems are not distinguished sharply. There is a continuous gradation in the firmness of metal-protein association, with the metalloenzymes at the extreme one side and the metal-enzyme complexes at the extreme other side of the scale.

The foregoing considerations imply that enzymes containing strongly

bound metal ions do not require the addition of metal ions for optimal enzymic activity. The activity of enzymes containing loosely bound metal ions is dependent on the free metal ion concentration in the assay mixture (MALMSTRÖM and ROSENBERG, 1959).

1.3.2 *Metal ion activation*

In the group of the metal-enzyme complexes the expression 'metal ion activation' was used to cover a number of effects (MALMSTRÖM, 1961). In some cases stabilization by metal ions was referred to as activation and only by a careful analysis of the effects observed one could distinguish between them (VALLEE, STEIN, SUMERWELL and FISCHER, 1959). Also the expression activation was used when metal ions influenced the rate of the enzymic reaction although the complex involved did not intrinsically participate in the catalytic mechanism, *e.g.* electrostatic effects. Furthermore metal ions can remove inhibitors and consequently it would appear that activation had occurred (COLEMAN, 1966). Only if the metal ion participates direct in the action mechanism of the enzyme this allows us to speak about metal ion activation: the metal ion acts as coenzyme or cofactor (MALMSTRÖM and ROSENBERG, 1959).

1.3.3 *The nature of the metal complex*

Once metal ion activation has been established, the question arises what kind of metal complex is involved. Three classes can be distinguished: 1) complex formation between substrate and metal: the metallosubstrate is the real substrate; 2) complex formation between enzyme and metal: the catalytically active form is the metal-enzyme complex and 3) complex formation with the enzyme-substrate complex.

Examples of the first class are: 3-phosphoglycerate kinase (ATP : 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3; LARSSON-RAŹNIKIEWICZ and MALMSTRÖM, 1961; LARSSON-RAŹNIKIEWICZ, 1964, 1967; COHN, 1963) and creatine kinase (ATP : creatine phosphotransferase, EC 2.7.3.2; COHN, 1963; MORRISON and UHR, 1966). The metal ion may be involved in formation of the ternary enzyme-metallosubstrate complex (SMITH, 1951) but this does not imply that

the metal acts as a bridge between substrate and enzyme. COHN and LEIGH (1962) concluded from spin relaxation measurements that the metal moiety of the metal-nucleotide substrate was not bound to the enzyme creatine kinase.

The second class of metal complexes (complex formation between metal ion and enzyme) is encountered with enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11; MALMSTRÖM, VÄNNGÅRD and LARSSON, 1958; COHN, 1963) and pyruvate kinase (ATP : pyruvate phosphotransferase, EC 2.7.1.40; MILDVAN, 1965, 1966). In this kind of complexes the metal ion usually is involved in bridge formation between enzyme and substrate.

In the third group (complex formation between metal ions and an enzyme-substrate complex) the metal ions exert a rate-activating effect through the higher velocity of breakdown of the enzyme-substrate complex in the presence of the metal ions (RABIN and CROOK, 1956).

The stability of the metal complexes formed is an important factor for metal effects. In polaric reactions the stability follows the 'IRVING-WILLIAMS sequence' (BRINTZINGER, 1965): $Ca^{2+} < Mg^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$, which is irrespective of the nature of the ligand and the number of ligand molecules involved (IRVING and WILLIAMS, 1953). Formation of transition-metal complexes is influenced by three interdependent factors, *viz.* ionic radius, electronegativity and 'd-state splitting' of the d-shell electronic orbitals when complexation occurs (WILLIAMS, 1959, 1961; VALLEE and COLEMAN, 1964).

1.4 PURPOSE OF THE PRESENT INVESTIGATION

Since metal ions were known to stimulate many enzymic reactions of the allantoin metabolism, the primary purpose of this study was to investigate these metal effects more in detail. Especially the effect of metal ions on the mechanism of activation and inactivation of allantoate amidohydrolase, the enzyme responsible for allantoate hydrolysis in the anaerobic pathway of degradation, was studied. The microbial metabolism of allantoin under aerobic conditions of growth has

been well elucidated. The metabolic pathway of allantoin degradation in microorganisms which grow under anaerobic conditions on allantoin was already proposed (VOGELS, 1963), but definite evidence for several of the suggested steps was lacking. Furthermore this study was undertaken in order to obtain conclusive evidence for the discrete steps in the anaerobic degradation pathway of allantoin.

CHAPTER 2

MATERIALS, METHODS AND DEFINITIONS

2.1 MATERIALS

The following chemicals and materials were used:
allantoin, purum (1); argininosuccinic acid, barium salt (2); L-asparagine. H_2O (3); N-carbamoylglycine (4); carbamoyl phosphate, dilithium salt (4); L-citrulline (4); L-cysteine, hydrochloride (5); diethanolamine (1); DEAE-(diethylaminoethyl)-cellulose (0.83 m.aequiv./g.) (6); EDTA (ethylenediaminetetraacetate, free acid and disodium salt) (7); glutamate dehydrogenase, suspension in glycerin (8); L-glutamine (7); GSH (glutathione, free acid, reduced) (4); glycine (5); glycylalanine ethylester (5); glycylglycine (5); glyoxylic acid. H_2O, sodium salt, purum (1); hippuric acid (5); α-ketoglutaric acid (8); methylurea (5); manganous sulfate. $4H_2O$ (7); $NADH_2$ (nicotinamide-adenine dinucleotide, reduced), disodium salt (8); L-ornithine, hydrochloride (1); oxaluric acid (4); PCMB (p-chloromercuribenzoate) (4); pyruvic acid, sodium salt (7); Sephadex G-25 (fine) and G-200 (140-400 mesh) (9); thioglycolic acid (5); triethanolamine (7); Tris (tris-(hydroxymethyl)-aminomethane) (7); urea (7); jack-bean urease (2); veronal (diethylbarbituric acid), sodium salt (7).

Other chemicals and materials used were of analytical grades. The above-mentioned chemicals and materials were obtained from the following sources:

(1) Fluka A.-G. Chemische Fabrik, Buchs SG, Switzerland.
(2) Sigma Chemical Company, Missouri, U.S.A.
(3) Union Chimique Belge, S.A., Belgium.
(4) Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.
(5) The British Drug Houses Ltd, Poole, England.
(6) Serva - Entwicklungslabor, Heidelberg, Germany.
(7) E.Merck A.-G., Darmstadt, Germany.

(8) C.F.Boehringer und Soehne G.m.b.H., Mannheim, Germany.
(9) Pharmacia, Uppsala, Sweden.

Sodium allantoate, sodium 4-methylallantoate, 5-aminohydantoin (hydrochloride), homoallantoic acid and the N-carbamoyl derivatives of L-asparagine, D-asparagine, L-alanine, D-alanine, L-aspartic acid and L-glutamine were obtained from Dr. G.D.VOGELS. Sodium ureidoglycolate was prepared according to the method of VALENTINE and WOLFE (1961), with the exception that the phosphate buffer (pH 7.0) was replaced by distilled water (TRIJBELS and VOGELS, 1966a).

2.2 QUANTITATIVE DETERMINATIONS

Ammonia was determined by nesslerization or by the glutamate dehydrogenase method. According to the latter method ammonia was determined by measuring at 340 mμ the dehydrogenation of $NADH_2$ in the presence of α-ketoglutarate (VOGELS, 1966). Nesslerization was only used when the sample contained no ureidoglycolate and/or glyoxylate, since both substances were responsible for a cloudiness obtained during the color reaction.

Urea was measured as ammonia after hydrolysis by jack-bean urease.

Allantoin, allantoate, ureidoglycolate and *glyoxylate* were determined according to a differential glyoxylate analysis (TRIJBELS and VOGELS, 1966a). The method could not be applied in the presence of cysteine or KCN (more than 0.1 μmole) or GSH (more than 0.3 μmole) in the sample because in the presence of these reducing substances lower intensities of color were obtained.

Protein was measured according to LOWRY *et al.* (1951).

Glycine was converted into formaldehyde according to CHRISTENSEN, RIGGS and RAY (1951). This compound, again, was determined by means of the method of ALEXANDER, LANDWEHR and SELIGMAN (1945). Glyoxylate did not interfere in this determination. However, several buffers were not suitable for incubation when this method was used, *e.g.* diethanolamine, triethanolamine and 2-amino, 2-methyl-propanediol 1,3, since during the determination the buffers themselves formed

formaldehyde or products which reacted like formaldehyde.

Manganese. Several methods were applied. The formaldoxime method (BARTLEY, NORTON and WERKHEISER, 1957) was mainly applied for qualitative demonstration of the presence of Mn^{2+} in enzyme preparations. This method could be used as a quantitative one after removal of protein from the sample. In most cases the electron paramagnetic resonance (EPR) technique was used.* Free manganese was measured by the intensity of its EPR spectrum. The EPR spectrum is given as a curve of the first derivative of the absorption against magnetic-field strength. It is assumed that the amplitude of the first derivative of the EPR absorption is a measure of the concentration (COHN and TOWNSEND, 1954). Measurements were performed with a Varian model V 4502 X-band spectrometer equipped with a Varian aqueous solution cell. No signal is observed when Mn^{2+} is bound in the form of a complex, because the spectral lines then obtained are so broad that detection is eluded (COHN, 1963). Very low concentrations ($<10^{-5}$M) of Mn^{2+} were measured by coupling the spectrometer to a Varian C 1024 time-averaging computer.

$^{54}Mn^{2+}$, obtained as $^{54}MnCl_2$ (0.115 mC/ml) from the Radiochemical Centre, Amersham, England, was measured with a Philips scintillation detector (PW 4119).**

2.3 PURIFICATION OF ALLANTOATE AMIDOHYDROLASE FROM STREPTOCOCCUS ALLANTOICUS

Acetone, precooled, was added dropwise to the crude cell-free extract at -10 to -15°. The protein fractions obtained between 0-53 % and between 53-58 % final acetone concentration were separated at 10 000 x *g* for 25 min at -20°. The latter fraction was combined with the protein fraction obtained between 48-53 % final acetone concentration by repre-

* We are greatly indebted to Dr. H. van Willigen, Laboratory of Physical Chemistry, University of Nijmegen, for his advice and cooperation in these experiments.

** We are indebted to Dr. J.H. Veerkamp, Laboratory of Biochemistry, University of Nijmegen, for his assistance in these measurements.

Table 2

Purification of allantoate amidohydrolase from *S. allantoicus*

	Total protein (mg)	Total activity (units)	Specific activity	Recovery (%)	Times purified
Crude cell-free extract	455*	1910	4.2	100	-
Acetone fractionation	51.3	1189	23.2	62	5.5
DEAE-cellulose chromatography	9.65	753	78	39	18.6
Sephadex G-200 gel filtration	2.56	536	210	28	50

* From *S. allantoicus* cells grown in 14 l allantoin-yeast extract medium for 24 h at 30°.

cipitation of the former fraction dissolved in 0.05 M Tris-HCl buffer (pH 7.5). The buffer used in the purification procedure always contained 1.7×10^{-4}M EDTA. The combined protein fractions, dissolved in the Tris buffer, were applied to a DEAE-cellulose column (32 cm x 1.8 cm), thoroughly equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). The adsorbed material was eluted stepwise with the same buffer containing an increasing NaCl concentration. The fraction containing the allantoate amidohydrolase activity was eluted with 0.35 M NaCl in the buffer and was dialyzed overnight against 0.05 M Tris-HCl buffer (pH 7.5), lyophilized and dissolved in 2.5 ml of the same buffer. This solution was applied to a column (78 cm x 2.1 cm) of Sephadex G-200 and the protein was eluted with the same Tris buffer. The active fractions were pooled and designated as purified enzyme. In Table 2 a typical result of the purification procedure is represented. The enzyme was purified 50-fold. The amount of nucleic acids in the purified material was less than 0.5 %, whereas crude enzyme preparations contained more than 20 %.

The same procedure applied to the enzyme from *A. allantoicus* resulted in a 40 times purified enzyme, but a recovery of only 10 % was obtained.

At a later stage of the investigation acetone fractionation was aban-

doned. After the stepwise DEAE-cellulose chromatography a gradient elution on DEAE-cellulose was performed and finally a gel filtration on Sephadex G-200.

The purification factor was between 30 - 50 times, resulting in enzyme preparations with a specific activity between 100 - 200.

2.4 CULTIVATION OF THE MICROORGANISMS AND PREPARATION OF CELL-FREE EXTRACTS

This was performed as given by VOGELS (1966). For disruption of the bacterial cells a MSE 500-W ultrasonic disintegrator was invariably used.

Extracts of plant materials were made as described by VOGELS, TRIJBELS and UFFINK (1966).

2.5 DEFINITIONS

In this study the following definitions will be used:

Stimulation of *enzyme activity* (*cofactor activity*): the enhancement of the enzymic activity by addition of certain substances at the otherwise complete activity test medium, *e.g.* addition of Mn^{2+} and GSH.

Activation of the *enzyme*: the enhancement of enzymic activity (measured under optimal conditions), which is a result of the pretreatment with certain substances and/or a change in pH of the enzyme solution. These substances are not involved in the catalytic action of the enzyme, *e.g.* NaBr, oxalate and EDTA.

Inactivation of the *enzyme*: the reversible decrease of enzymic activity, which is a result of the addition of certain substances.

Instability of the *enzyme*: the reversible decrease of enzymic activity, which occurs spontaneously (without addition of substances).

Denaturation: the irreversible loss of enzymic activity.

Terms like *increase, decrease, enhancement, diminution, extent, inhibition, etc.*, will have no special meaning.

One *unit* of *enzyme activity* is defined as the amount which catalyzes the transformation of one μmole substrate per minute.

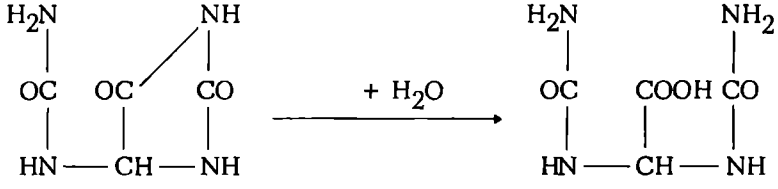
Specific activity is expressed in units per mg protein.

Buffer molarities refer to the concentrations of the buffering substances.

CHAPTER 3

THE EFFECT OF BIVALENT CATIONS ON THE ACTIVITY OF ALLANTOINASES FROM PLANTS, BACTERIAL AND ANIMAL SOURCES

Allantoinase (allantoin amidohydrolase, EC 3.5.2.5) catalyzes the reaction:



ALLANTOIN ALLANTOIC ACID

This enzyme is widespread in nature. Its occurrence was reported in animals (FOSSE and BRUNEL, 1929; LASKOWSKI, 1951; BRODSKY *et al.*, 1965; TRIJBELS, 1967), higher plants (STEWARD and POLLARD, 1957; VAN DER DRIFT and VOGELS, 1966), algae (AMMANN and LYNCH, 1964), basidiomycetes (BRUNEL, 1936) and microorganisms (VOGELS, 1963; LEE and ROUSH, 1964). A review of properties of allantoinases from different sources was given recently by VOGELS (1963) and TRIJBELS (1967). An extensive investigation by VOGELS *et al.* (1966) yielded information on purification and enzymic properties of allantoinases from nine different sources.

In this Chapter we shall discuss the effects of bivalent cations and reducing substances on the activity of these nine enzymes (VOGELS and VAN DER DRIFT, 1966).

Table 3

Effect of Mn^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+} and EDTA on the activities of allantoinases

Allantoinase activities were determined at 30° in mixtures containing, per ml, 33 µmoles allantoin, 160 µmoles diethanolamine-HCl buffer (pH 7.7) and the indicated amounts of purified allantoinase and cations or EDTA. In the experiments with *S. allantoicus*, *A. allantoicus* and *E. coli*, moreover, 0.07 µmole $MnSO_4$ and 8.7 µmoles GSH were present per ml.

Organism	Purified allantoinase (µg/ml)	v*	Activities * (%)									
			$MnSO_4$		$ZnSO_4$		$CoSO_4$		$CdSO_4$		EDTA	
			5x 10^{-3} M	2.5x 10^{-4} M	5x 10^{-3} M	2.5x 10^{-4} M	5x 10^{-3} M	2.5x 10^{-4} M	5x 10^{-3} M	2.5x 10^{-4} M	5x 10^{-3} M	2.5x 10^{-4} M
S. allantoicus	17	150	-	-	36	53	35	93	43	96	35	35
A. allantoicus	20	117	-	-	15	54	32	101	22	106	7	8
E. coli	22	116	-	-	12	83	16	94	13	101	6	9
P. acidovorans	1	54	8	47	0	0	0	51	0	0	108	98
P. fluorescens	9	12	0	15	0	4	0	44	0	0	104	100
Frog liver	4	7.6	90	90	8	42	54	103	13	40	100	100
Goldfish liver	45	6.3	85	90	0	16	51	86	0	4	105	101
Ph. hysterinus	250	54	185	172	75	111	116	115	101	123	101	100
G. hispida	560	41	195	229	98	120	110	115	107	120	105	103

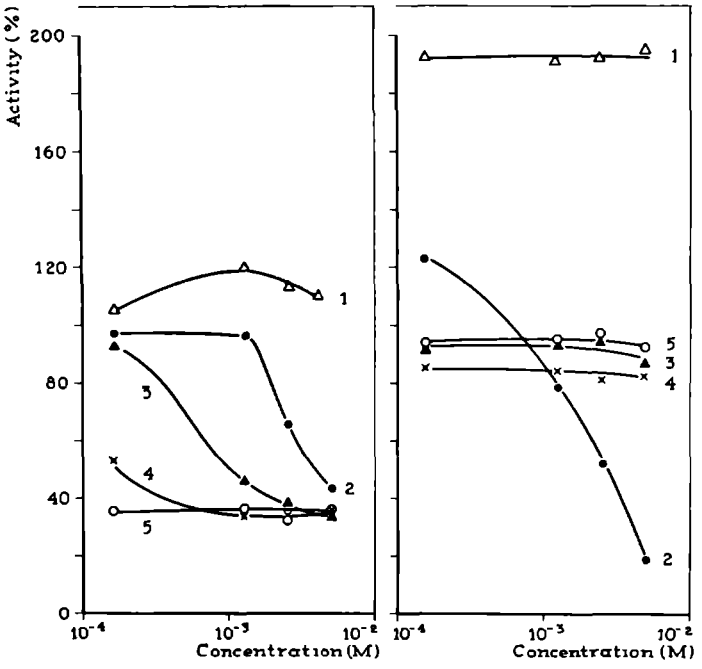
* v (mµmoles allantoin converted per ml per min) was measured in the absence of additions, and the activity in the presence of these compounds is given in % of this value.

3.1 INFLUENCE OF BIVALENT CATIONS ON THE ACTIVITY OF ALLANTOINASES

The allantoinase from mung beans was strongly inhibited by Cu^{2+} and slightly by Mn^{2+}. Hg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, $Fe^{2+}$and EDTA (all 10^{-3}M) were without effect (NAGAI and FUNAHASHI, 1961). The enzyme from bakers' yeast was only slightly inhibited by metal ions and no direct evidence was found for a metal ion as cofactor, although after dialysis 65 % of the activity was lost (LEE and ROUSH, 1964). VOGELS (1963) reported the inhibition of allantoinases from *S.allantoicus* and *A.allantoicus* by Hg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, $Co^{2+}$and EDTA (10^{-3}M and 10^{-4}M). Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Ni^{2+} and Fe^{3+} did not exhibit an effect at the concentrations tested. Mn^{2+} enhanced the activity of crude enzyme preparations 30 % and 100 %, respectively.

In Table 3 are represented the effects of Mn^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+} and EDTA on nine allantoinases, purified according to VOGELS *et al.* (1966). Zn^{2+}, Co^{2+} and Cd^{2+} reduced the activity of all allantoinases, except those from higher plants which were slightly stimulated. The enzymes from pseudomonads were strongly inhibited by the cations tested, while the enzymes from animal livers, *S.allantoicus*, *A.allantoicus* and *E.coli* were less inhibited. Mn^{2+} strongly reduced the activity of the Pseudomonas enzymes, slightly those from animal livers, but stimulated the enzymes from higher plants about twice. This was, however, no general observation, because the enzymes from wheat and gherkin were inhibited by 10^{-4}M Mn^{2+}.

The effect of Mn^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+} and EDTA on the activity of allantoinase from *S.allantoicus* is given in Figs. 1a and 1b. Activity was measured both in the presence (Fig.1a) and absence (Fig.1b) of 7×10^{-5}M Mn^{2+}. In the presence of Mn^{2+} the enzymic activity was lowered to about one third of the original activity by Co^{2+}, Zn^{2+} and EDTA, whereas in the absence of Mn^{2+} only a slight decrease was observed. Since EDTA, Co^{2+} and Zn^{2+} inhibited to the same extent in the presence of Mn^{2+} and were only inhibitory in the presence of this cation, it was probable that inhibition was due to replacement of Mn^{2+} from the enzyme molecule. Cd^{2+} seemed to exert a different kind of inhibition, since at higher concentrations Cd^{2+} inhibited strongly in the



Figures 1a and 1b

Effects of Mn^{2+} (1), Cd^{2+} (2), Co^{2+} (3), Zn^{2+} (4) and EDTA (5) in different concentrations on the allantoinase activity of *S.allantoicus* in the presence (Fig.1a) and absence (Fig.1b) of 7 x 10^{-5}M $MnSO_4$. Tests were performed as given in Table 3, except that in the second experiment $MnSO_4$ and GSH were omitted from the medium. The activities are expressed in % of the activity without additions.

absence of Mn^{2+}, while in the presence of Mn^{2+} a protection against this inhibiting effect took place.

Manganous ions were not essential for enzymic activity of *S.allantoicus* allantoinase, but enhanced the activity about twice. Chelating agents, such as EDTA and 1,10-phenanthroline, did not decrease the enzymic activity in the absence of Mn^{2+} ions. With the enzymes from *A.allantoicus* and *E.coli* similar results were obtained: without addition of Mn^{2+} and GSH the activity was about 10 % and 20 %, respectively, of those observed in the presence of these compounds.

3.2 EFFECT OF REDUCING SUBSTANCES AND KCN

An inhibition of allantoin degradation by cell suspensions of unidentified Pseudomonas species in the presence of KCN was observed by BACHRACH (1957). The activity of mung bean allantoinase (NAGAI and FUNAHASHI, 1961) and soy bean allantoinase (LEE and ROUSH, 1964) was not influenced by 10^{-3}M and 10^{-2}M KCN, respectively, but the enzyme from yeast was strongly (90%) inhibited by 10^{-2}M KCN (LEE and ROUSH, 1964). Allantoinases from yeast, soy beans and *Phaseolus hysterinus* Dur. (VAN DER DRIFT and VOGELS, 1966) were strongly inhibited by cysteine and GSH, while the enzymes from *S. allantoicus* and *A. allantoicus* were stimulated by cysteine and GSH (VOGELS, 1963).

The effect of cysteine, thioglycolate, GSH and KCN on nine allantoinases is shown in Table 4. On the basis of these results allantoinases

Table 4

Effect of reducing substances and KCN on the enzymic activities of allantoinases

Allantoinase activities were determined at 30° in mixtures containing, per ml, 39 µmoles allantoin, 200 µmoles diethanolamine-HCl buffer (pH 7.7) and the indicated amounts of purified allantoinase and of reducing substances. In the experiments with *S. allantoicus*, *A. allantoicus* and *E. coli* 0.1 µmole $MnSO_4$ was added together with the reducing substances and allantoin. v was measured in the absence of reducing substances and KCN. The other values are expressed in % of this value.

Organism	Purified allantoinase (µg/ml)	v*	Activities (%)				
			Cysteine 10^{-2}M	Thioglycolate 10^{-2}M	Glutathione 10^{-2}M	KCN 2.5x 10^{-3}M	KCN 2.5x 10^{-4}M
S. allantoicus	72	409	181	180	148	100	95
A. allantoicus	24	14	1140	650	550	310	-
E. coli	38	19	1100	950	910	130	100
P. acidovorans	1.1	60	100	100	100	95	100
P. fluorescens	10	12	106	116	115	100	100
Frog liver	4.5	7.6	25	60	100	39	76
Goldfish liver	52	7.3	33	85	100	30	77
Ph. hysterinus	290	62	0	66	66	83	87
G. hispida	640	47.7	7	68	86	90	95

* v in mµmoles allantoin converted per ml per min.

could be divided into three groups: one group (*S. allantoicus*, *A. allantoicus* and *E. coli* allantoinases) was stimulated by reducing substances and KCN which can also act as a reducing agent to open disulfide links in proteins. The second group (Pseudomonas allantoinases) was not influenced by these compounds, and the third (allantoinases from animals and plants) was inhibited.

Iodoacetate tested under the conditions given in Table 4 did not inhibit any of the enzymes. Mung bean allantoinase was not affected either by iodoacetate (NAGAI and FUNAHASHI, 1961), but yeast allantoinase was inhibited at a concentration of about 10^{-2}M; however, it was stated that no essential SH-groups were present in the yeast enzyme (LEE and ROUSH, 1964).

Table 5

Stimulation and inhibition of some allantoinases by manganous ions and reducing substances

Allantoinase activities were measured at 30° in mixtures containing, per ml, in the first three experiments, 160 μmoles diethanolamine-HCl buffer (pH 7.7), 33 μmoles allantoin and the indicated amounts of purified allantoinase, which were added to the otherwise complete mixture. $MnSO_4$ and GSH were present in amounts of 1.25 and 8.7 μmoles, respectively. In the tests with allantoinases from higher plants there were present, per ml, 200 μmoles diethanolamine-HCl buffer (pH 7.7), 39 μmoles allantoin and the indicated amounts of purified allantoinase added to the otherwise complete mixture. $MnSO_4$ and cysteine were present in amounts of 0.08 and 10 μmoles, respectively.

Organism	Purified allantoinase (μg/ml)	v*	Activities* (%)				
			$+Mn^{2+}$	+ GSH	$+Mn^{2+}$ +GSH	+Cysteine	$+Mn^{2+}$ + Cysteine
S. allantoicus	34	71	153	119	228		
A. allantoicus	20.6	6.6	485	124	1330		
E. coli	8.9	3.8	190	165	740		
Ph. hysterinus	290	54	214			0	123
G. hispida	425	41.4	262			0	179

* v in mμmoles allantoin converted per ml per min. The activities measured in the presence of Mn^{2+} and reducing substances are given in % of this value.

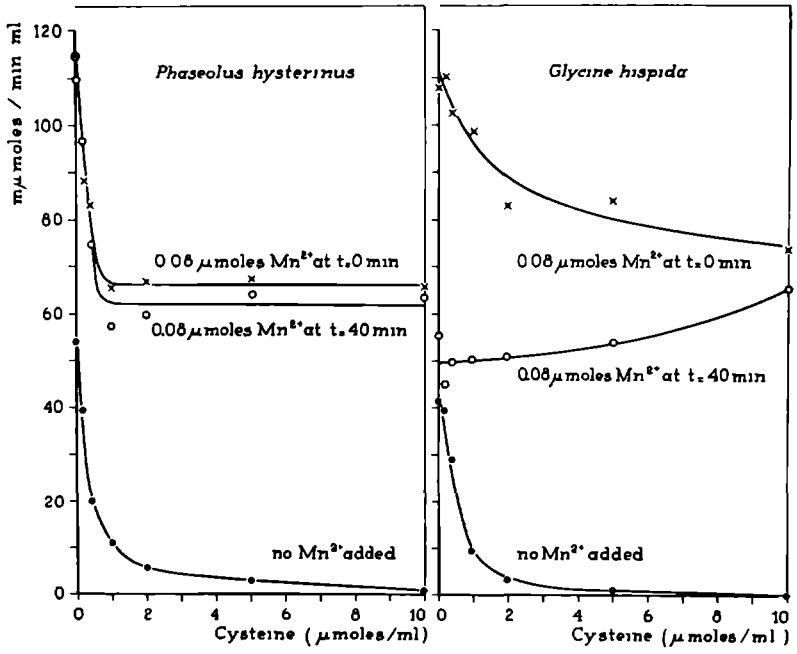
3.3 INTERACTION OF MANGANOUS IONS AND REDUCING SUBSTANCES

Surveying the effects obtained by Mn^{2+} ions and reducing substances it appeared that at least four groups of allantoinases could be distinguished. Stimulation by both Mn^{2+} and reducing substances was observed with the enzymes from *S. allantoicus*, *A. allantoicus* and *E. coli*. Allantoinases from Pseudomonas species were inhibited by Mn^{2+} but not affected by reducing substances. The enzymes from animal livers were inhibited both by Mn^{2+} and reducing substances and finally allantoinases from plants were stimulated by Mn^{2+} and inhibited by reducing substances.

Inhibition by cysteine of the animal allantoinases was not influenced by addition of Mn^{2+} but a distinct interaction of reducing compounds and Mn^{2+} was obtained with the enzymes from higher plants, *S. allantoicus*, *A. allantoicus* and *E. coli* (Table 5). When both substances were added simultaneously the observed activity was higher than could be expected from results with the additions tested separately. The strong inhibition of plant allantoinases by cysteine was counteracted by the addition of Mn^{2+} (Figs. 2a and 2b). In the absence of Mn^{2+} almost complete inhibition occurred at a cysteine concentration of 2×10^{-3} M, but in the presence of Mn^{2+} this inhibition was only 40 % and 30%, even in the presence of 10^{-2}M cysteine. The same effect on cysteine-inhibited allantoinases was observed when Mn^{2+} was added 40 min after the start of the experiment. The activity of soy bean allantoinase was not restored completely under these conditions. Probably this was due to a decreasing sensitivity of the enzyme to the stimulating effect of Mn^{2+} on preincubation with allantoin (Fig.3). Preincubation of the enzyme with Mn^{2+}, however, resulted in a more active enzyme.

3.4 INHIBITION OF ALLANTOINASE FROM STREPTOCOCCUS ALLANTOICUS BY Cd^{2+}. EFFECT OF Mn^{2+} AND GLUTATHIONE

It appeared from Fig.1b that the inhibition of the enzyme from *S. allantoicus* by Cd^{2+} was due to another mechanism than inhibition by Zn^{2+}, Co^{2+} and EDTA. The latter process was regarded as a removal



Figures 2a and 2b

Inhibition of allantoinases from *Phaseolus hysterinus* (Fig.2a) and soy beans (Fig. 2b) by different amounts of cysteine, and counteraction of this inhibition by Mn^{2+}. The incubation mixtures at 30° contained, per ml, 200 μmoles diethanolamine-HCl buffer (pH 7.7), 39 μmoles allantoin, the indicated amounts of cysteine and 0.29 or 0.42 mg purified allantoinase from *Phaseolus hysterinus* or soy beans, respectively. Two series of incubation mixtures were tested, to one series 0.08 μmole $MnSO_4$ was added together with the allantoinases at the start of the experiment. In the second series the velocity was followed for 40 min in the absence of Mn^{2+}. Then, 0.08 μmole $MnSO_4$ was added, and the activity was measured during a prolonged incubation time of 40 min.

of added Mn^{2+} from the enzyme molecule. Since Cd^{2+} also inhibited strongly without extra addition of Mn^{2+}, another process must be involved.

In order to study the mechanism of the inhibition by Cd^{2+} this compound was added to the enzyme from *S.allantoicus* in the presence of the cofactors Mn^{2+} and GSH (Table 6). The order of addition was varied. Cd^{2+}, added separately or together with Mn^{2+}, strongly inhibited the reaction (Expts. 4, 7 and 8). In combination with GSH inhibition only took

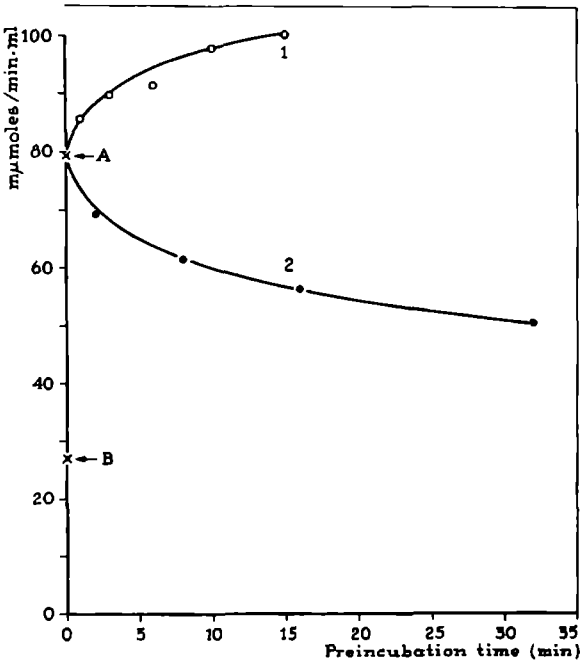


Figure 3

Effect of preincubation with Mn^{2+} (1) or allantoin (2) on the enzymic activity of allantoinase from soy beans. Preincubation with Mn^{2+} was performed in a mixture containing, per ml, 180 μmoles diethanolamine-HCl buffer (pH 7.7), 0.1 μmole $MnSO_4$ and 3.3 mg purified allantoinase from soy beans. At the indicated time intervals an aliquot (0.1 ml) of the mixture was added to 1 ml of an allantoin solution in diethanolamine buffer containing a second amount of Mn^{2+}. The resulting incubation mixtures at 30° contained, per ml, 45 μmoles allantoin, 0.1 μmole $MnSO_4$, 220 μmoles diethanolamine-HCl buffer (pH 7.7) and 0.3 mg purified enzyme. Preincubation with allantoin was performed in a mixture containing, per ml, 45 μmoles allantoin, 220 μmoles diethanolamine-HCl buffer (pH 7.7) and 0.3 mg purified enzyme. The allantoin conversion was measured in this mixture and at the indicated time intervals $MnSO_4$ was added (0.01 ml to 1 ml preincubation mixture). The final concentration of Mn^{2+} was 10^{-4}M. The velocity of the allantoinase reaction was measured again, and the values obtained are represented in the figure. Points A and B indicate the velocities measured when Mn^{2+} was added together with allantoin at the start, or when these ions were omitted from the medium, respectively.

place if Cd^{2+} was added before GSH (Expts. 10-12, 14 and 25); GSH protected the enzyme against the inhibition by Cd^{2+} (Expts. 9,13,15,16 and 24). The same did allantoin (Expts. 19 and 23), but at the same time the

Table 6

Stimulation of *S. allantoicus* allantoinase by Mn^{2+} and GSH; inhibition by Cd^{2+}. Effect of the order of additions

To 0.5 ml cell-free extract of *S. allantoicus*, containing 110 μmoles diethanolamine-HCl buffer (pH 7.7) and 98 μg protein, there were added in the order given below: (a) 0.2 ml of a solution containing 13 μmoles GSH and 53 μmoles diethanolamine-HCl buffer (pH 7.7); (b) 0.1 ml containing 1.9 μmole $MnSO_4$; (c) 0.1 ml containing 1.9 μmole $CdSO_4$ and (d) 1 ml containing 50.6 μmoles allantoin and 230 μmoles diethanolamine-HCl buffer (pH 7.7). The final volume was 1.9 ml. When one of the additions was omitted it was replaced by the same volume of water. All incubations were performed at 30°. In Expts. 1-16 there was an interval of 5 min between each addition, and the velocity was measured 10, 20 and 30 min after addition of the allantoin solution. In the Expts. 17-25 there was also an interval of 5 min after the addition of the first compound to the extract. Then, allantoin was added and the velocity (v_1) was measured 5 and 10 min after this addition. A second compound was added 15 min after the addition of allantoin, and the velocity (v_2) was measured 5, 15 and 25 min after this addition. v is expressed in mμmoles allantoin converted per min per ml.

Expt.	Order of addition					v	v_1	Addition at t = 15	v_2
	(1)	(2)	(3)	(4)	(5)				
1	Extract	-	-	-	Allantoin	45			
2	Extract	-	-	GSH	Allantoin	49			
3	Extract	-	-	Mn^{2+}	Allantoin	66			
4	Extract	-	-	Cd^{2+}	Allantoin	6			
5	Extract	-	GSH	Mn^{2+}	Allantoin	92			
6	Extract	-	Mn^{2+}	GSH	Allantoin	93			
7	Extract	-	Cd^{2+}	Mn^{2+}	Allantoin	2			
8	Extract	-	Mn^{2+}	Cd^{2+}	Allantoin	12			
9	Extract	-	GSH	Cd^{2+}	Allantoin	52			
10	Extract	-	Cd^{2+}	GSH	Allantoin	8			
11	Extract	Cd^{2+}	Mn^{2+}	GSH	Allantoin	2			
12	Extract	Cd^{2+}	GSH	Mn^{2+}	Allantoin	10			
13	Extract	Mn^{2+}	GSH	Cd^{2+}	Allantoin	119			
14	Extract	Mn^{2+}	Cd^{2+}	GSH	Allantoin	22			
15	Extract	GSH	Cd^{2+}	Mn^{2+}	Allantoin	112			
16	Extract	GSH	Mn^{2+}	Cd^{2+}	Allantoin	114			
17	Extract	-	Allantoin				45	GSH	50
18	Extract	-	Allantoin				45	Mn^{2+}	51
19	Extract	-	Allantoin				45	Cd^{2+}	36
20	Extract	GSH	Allantoin				52	Mn^{2+}	65
21	Extract	Mn^{2+}	Allantoin				67	GSH	81
22	Extract	Cd^{2+}	Allantoin				4	Mn^{2+}	4
23	Extract	Mn^{2+}	Allantoin				66	Cd^{2+}	47
24	Extract	GSH	Allantoin				52	Cd^{2+}	59
25	Extract	Cd^{2+}	Allantoin				5	GSH	8

substrate counteracted stimulation by Mn^{2+} (Expts. 18 and 20). Changes in the order of addition of Mn^{2+} and Cd^{2+} (Expts. 7 and 8) or of Mn^{2+} and GSH (Expts. 5 and 6) had only a slight effect on the enzymic activity. Again it was noticed that the stimulation by Mn^{2+} and GSH together was larger than would be expected (see also Table 5).

3.5 INHIBITION BY PHOSPHATE

The first observation on inhibition of allantoinase by phosphate was reported by LEE and ROUSH (1964), who measured the activity of yeast allantoinase in 0.1 M phosphate buffer. These authors did not state the pH of their assay mixtures. NAGAI and FUNAHASHI (1961) and FRANKE *et al.* (1965) did not report an inhibiting effect of phosphate buffers on allantoinases from mung beans and soy beans, respectively. Both authors used phosphate buffers in the determination of the optimal pH. VOGELS *et al.* (1966) observed that phosphate inhibited the activity of allantoinases from frog and goldfish liver, soy beans and *Phaseolus hysterinus*. Stronger inhibition of the enzyme occurred when the pH of the incubation mixture was lowered. At pH 7.5 the enzymes from frog and goldfish liver were inhibited 50 % and 60 %, respectively, whereas the plant enzymes were no longer inhibited. The enzyme from Pseudomonas species was not inhibited either at pH values between 6.7 and 7.8.

At pH 7.7 only the enzymes from *S.allantoicus*, *A.allantoicus*, *E.coli* and animal livers were inhibited by phosphate buffers. The effect of the molarity of the phosphate buffers is shown in Fig. 4. Mn^{2+} was present in the experiments performed with bacterial allantoinases; the effect of Mn^{2+} on the inhibition of *S.allantoicus* and plant allantoinases is represented in Fig. 5. The bacterial enzyme was only slightly inhibited in the absence but rather strongly in the presence of Mn^{2+}. In the presence of Mn^{2+} the activity was lowered to the same value as that found in its absence. Probably this inhibition was due to a removal of Mn^{2+} by combination with $H_2PO_4^-$. Allantoinases from *A.allantoicus* and *E.coli* yielded similar results. In contrast to these allantoinases the enzymes from plants were strongly inhibited by phosphate even in

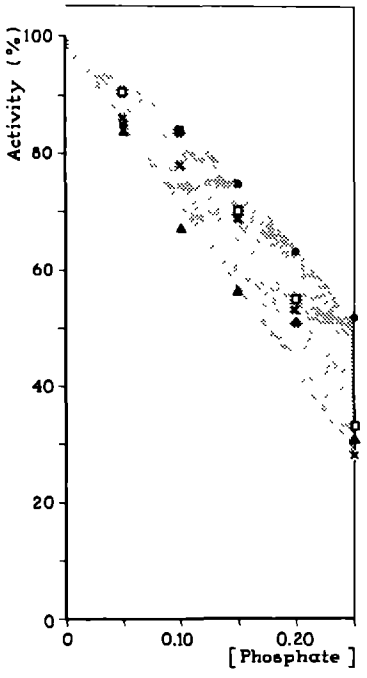


Figure 4

Inhibition of allantoinases from *S.allantoicus* (●), *A.allantoicus* (×), *E.coli* (▲) and frog liver (□) by phosphate buffers (pH 7.7) with increasing molarities. Allantoin solutions in Tris-HCl buffer (pH 7.7) and in KH_2PO_4-Na_2HPO_4 buffer (pH 7.7) were mixed to obtain the indicated molarities of phosphate buffer. There were present, per ml incubation mixture, 42 µmoles allantoin, 0.1 µmole $MnSO_4$, 5.5 µmoles GSH and 250 µmoles buffer (composed of Tris and phosphate buffer). $MnSO_4$ and GSH were absent in the experiments with frog liver allantoinase. The amount of purified allantoinase, per ml, was 21 µg *(S.allantoicus)*, 26 µg *(A.allantoicus)*, 51 µg *(E.coli)* and 13.5 µg (frog liver). The velocities measured in the absence of phosphate buffers were 177, 91.5, 153 and 12.2 mµmoles allantoin converted per min per ml, respectively.

the absence of Mn^{2+} (Fig.5): 75 % inhibition was measured in 0.18 M phosphate buffer (pH 6.9). A similar inhibition was observed for animal liver allantoinases. This phosphate inhibition cannot be explained by removal of Mn^{2+} or another bivalent cation which acted as cofactor, since EDTA did not change the activity of the plant and animal enzymes (Table 3).

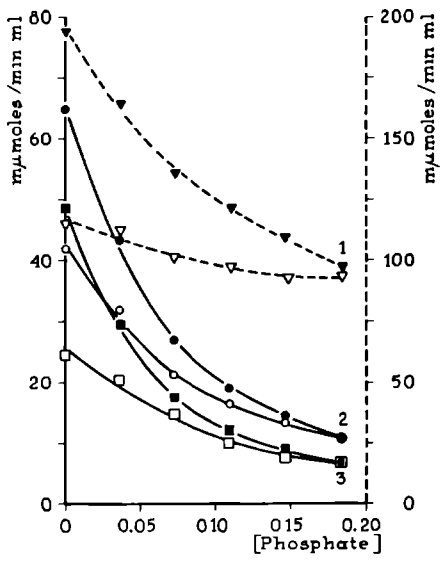


Figure 5

Inhibiting effect of phosphate buffer (pH 6.9) on the allantoinase activities of *S. allantoicus* (1), *Phaseolus hysterinus* (2) and soy beans (3) in the presence (closed symbols) and absence (open symbols) of Mn^{2+}. Allantoin solutions in Tris-HCl buffer (pH 6.9) and KH_2PO_4-Na_2HPO_4 buffer (pH 6.9) were mixed to obtain the indicated molarities of phosphate buffer. The incubation mixtures at 30° contained, per ml, 183 μmoles buffer (mixtures of Tris and phosphate buffer), 46 μmoles allantoin and 114 μg (cell-free extract of *S. allantoicus*), 300 μg (soy beans) or 340 μg (*Phaseolus hysterinus*) protein. In one series of experiments (closed symbols), moreover, 0.1 μmole Mn^{2+} was present per ml incubation mixture.

3.6 DISCUSSION AND CONCLUSIONS

Recently an attempt was made to subdivide allantoinases from different sources in groups (VOGELS *et al.*, 1966). Although a number of properties (pH-activity curve, K_m, activation energy and stability on storage, heating, urea treatment and acid-pretreatment) was determined, no satisfactory classification of the enzymes could be made on this basis. Nor did the specificity of the allantoinases for allantoin derivatives and (+)- and (-)-allantoin allow a clear-cut subdivision. A classification into four groups of allantoinases could be made by

using the effects of bivalent cations and reducing substances (Table 7). The first group (I) includes the enzymes from *S. allantoicus*, *A. allantoicus* and *E. coli*. These organisms could use allantoin as the main source of carbon, nitrogen and energy for growth under anaerobic conditions. These allantoinases were not stereospecific, in contrast to the other allantoinases, and were activated by Mn^{2+} and reducing substances. The second group (II) was formed by the enzymes from Pseudomonas species which only used allantoin for growth under aerobic conditions. These enzymes were inhibited by Mn^{2+} and other bivalent cations, whereas reducing compounds had no effect on the activity. The third group (III) consisted of the enzymes from two animal livers. These allantoinases were slightly inhibited by Mn^{2+} and strongly by other bivalent cations. Reducing substances also inhibited these allantoinases. Finally, the fourth group (IV) was formed by enzymes from higher plants; these enzymes were stimulated by Mn^{2+} and other cations but strongly inhibited by reducing compounds.

This classification, however, did not seem to be complete; as mentioned before (3.1), allantoinases from two other plants, *viz.* wheat and gherkin, were inhibited by Mn^{2+}. Furthermore no activation during acid-pretreatment occurred with these two enzymes, in contrast to the

Table 7

Some properties of allantoinases

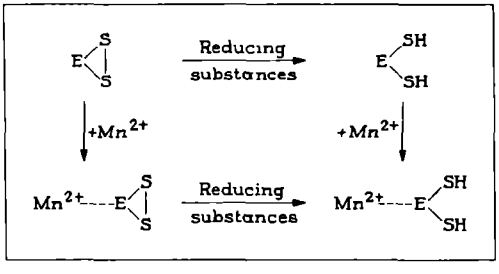
Group	Organism	Optical specificity*	Effect of some additions				Acid-pre-treatment*
			Mn^{2+}	Zn^{2+}	Reducing substances	Phosphate buffer (pH 7)	
I	*S. allantoicus* *A. allantoicus* *E. coli*	Aspecific	Stimulation	None	Stimulation	Slight or no inhibition	Decrease of activity
II	*P. acidovorans* *P. fluorescens*	Specific	Strong inhibition	Strong inhibition	None	None	Decrease of activity
III	Frog liver Goldfish liver	Specific	Inhibition	Strong inhibition	Inhibition	Strong inhibition	Decrease of activity
IV	*Ph. hysterinus* *G. hispida*	Specific	Stimulation	Slight stimulation	Strong inhibition	Inhibition	Activation

* VOGELS *et al.*, 1966.

enzymes from two beans (VOGELS *et al.*, 1966). Allantoinase from *Penicillium notatum* was inhibited by 10^{-4}M Mn^{2+} (24% inhibition) and stimulated by 10^{-2}M GSH (24 % stimulation). Yeast allantoinase was inhibited by cysteine, but not stimulated by Mn^{2+} (LEE and ROUSH, 1964).

Mn^{2+} ions acted as cofactors for the enzymes from groups I and IV but were not essential, since chelating substances did not eliminate the enzymic activity. Inhibition by Mn^{2+} occurred with the enzymes from groups II and III. Reducing substances (cysteine, thioglycolate, GSH) enhanced the enzymic activity of allantoinases of group I, while the enzymes from groups III and IV were inhibited. The inhibition by reducing substances in group IV could be counteracted by Mn^{2+}.

An explanation of the differences observed among the allantoinases could be given by accepting four structural configurations with different activities for all these enzymes (Scheme 3). In group I all four configurations were active, but the Mn^{2+}-bound reduced form was the most active one. In group II only the two Mn^{2+}-free configurations were active and their activities were equal. In group III the Mn^{2+}-free oxidized form was most active, while the Mn^{2+}-bound oxidized form was about 10 % less active. Both reduced forms of the enzyme were much less active. In group IV highest activity was displayed by the Mn^{2+}-bound oxidized form; the Mn^{2+}-bound reduced form and the Mn^{2+}-free oxidized form had a lower activity (about 50 %), while the Mn^{2+}-free reduced form was catalytically inactive. In this classification allantoinase from *Pen.notatum* had highest activity in the Mn^{2+}-free reduced configuration.



Scheme 3
Four possible configurations of allantoinases

Sometimes, especially for group IV, other bivalent cations (Zn^{2+}, Co^{2+} or Cd^{2+}) could replace Mn^{2+} as a cofactor. Inhibition of the enzyme from *S.allantoicus* by Cd^{2+} consisted in binding of Cd^{2+} to the oxidized enzyme, while Cd^{2+}-binding to the reduced enzyme did not influence the enzymic activity.

The location of the thiol groups and of the Mn^{2+}-binding groups on the enzyme molecule is unknown. It is likely that these groups are at or near the active site of the enzyme, since preincubation with allantoin reduced the stimulating effect of Mn^{2+} on the enzymes from soy beans and *S.allantoicus*. Furthermore, the inhibition by Cd^{2+} on the latter enzyme was almost completely abolished upon preincubation of the enzyme with substrate.

Since Mn^{2+}-enzyme interactions were obtained with allantoinases, attempts were made to distinguish between the possibilities given in Chapter 1 (1.3), *viz.* allantoinase is a metalloenzyme or a metal-enzyme complex. Allantoinase from *P. acidovorans* (specific activity 1.5) was purified 60 times by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography and gel filtration on Sephadex G-200 (unpublished results, VOGELS). The specific activity of the purified enzyme material was 90. Manganese was determined by means of emission spectrography* and amounted to 20 $\pm$ 5 p.p.m. The molecular weight of this allantoinase was about 60 000 as estimated by gel filtration. This would mean that less than 2.5 % of the enzyme molecules contained a Mn^{2+} ion. Therefore, this enzyme and perhaps also the other allantoinases cannot be considered a metalloenzyme.

* Emission spectrography was performed by Dr. P.W.J.M. Boumans and F.J.M.J. Maessen, Laboratory of Analytical Chemistry, University of Amsterdam.

CHAPTER 4

MECHANISM OF THE REACTION CATALYZED BY ALLANTOATE AMIDOHYDROLASE

Allantoate is the first product formed in the degradation pathway of allantoin. For the breakdown of allantoate two pathways are known: 1) the allantoicase-catalyzed hydrolysis (1.1.2, Scheme 1) and 2) the allantoate amidohydrolase-catalyzed hydrolysis (1.1.2, Scheme 2). The first pathway yields one mole of urea and one mole of ureidoglycolate from one mole of allantoate. This mechanism was studied by TRIJBELS (1967) and is operative in animal organs and in microorganisms which grow on allantoin under aerobic conditions, except *P. acidovorans*. The second pathway was demonstrated by VOGELS (1963, 1966) in microorganisms which grow on allantoin only under anaerobic conditions. This pathway is also present in *P. acidovorans* (TRIJBELS and VOGELS, 1966a). Ureidoglycolate is a common intermediate in both routes of allantoate hydrolysis. In the second pathway no urea but two moles of ammonia and one mole of carbon dioxide were formed from allantoate (VOGELS, 1963, 1966). The intermediate position of ureidoglycolate was supposed on the basis of the observation that ureidoglycolate was broken down to glyoxylate and urea by cell-free extracts of the organisms. Ureidoglycolate production from allantoate, however, was not measured at that time, since no analytical determination for this substance was available.

Since detailed information on the degradation of allantoate along the second pathway was scarce, we started an investigation on the degradation route in *S. allantoicus*. This microorganism grows only under anaerobic conditions on allantoin and follows the second degradation route (VOGELS, 1963). More information on the role of the enzyme allantoate amidohydrolase was also important considered in the light of the 'acid-activation' of this enzyme. This phenomenon will be discussed in Chapter 6.

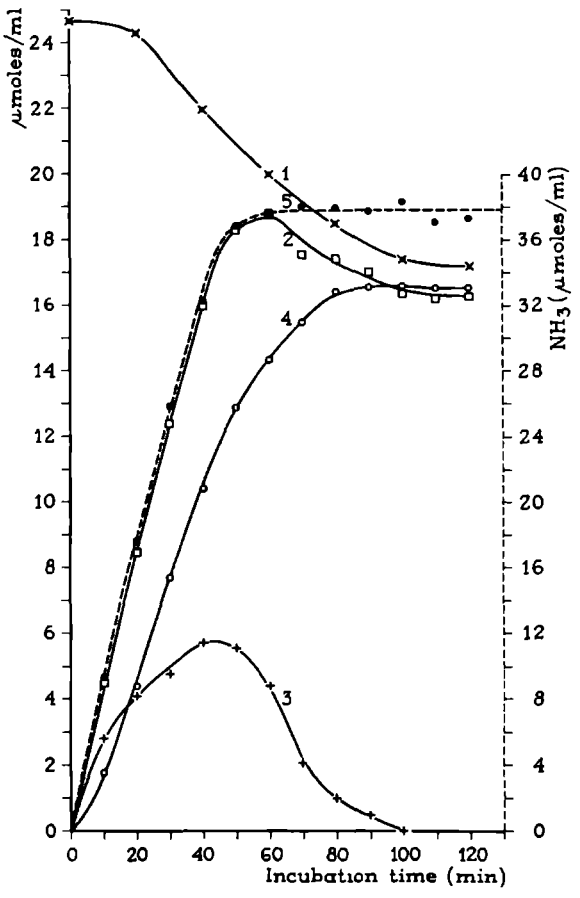


Figure 6

Degradation of allantoate by cell-free extract from *S.allantoicus*. 0.2 ml enzyme solution containing 0.56 mg protein and 34 mµmoles EDTA in 0.05 M Tris-HCl buffer (pH 7.5) was treated with 0.6 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.95) (cf. Chapter 6). After 30 min at 30° 2 ml buffered substrate solution were added. The final incubation mixture contained, per ml, 24.6 µmoles sodium allantoate, 4.23 µmoles GSH, 0.11 µmole $MnSO_4$, 93 µmoles diethanolamine-HCl buffer (pH 8.8) and 0.2 mg protein. Incubation was at 30°. Sum of glyoxylate and its acid-labile derivatives (1), ureidoglycolate + glyoxylate (2), ureidoglycolate (3) and glyoxylate (4) were determined according to the differential glyoxylate analysis. Ammonia (5) was measured according to the glutamate dehydrogenase method.

4.1 THE PRODUCTION OF AMMONIA FROM ALLANTOATE

The first task was to prove unequivocally that the mechanism responsible for hydrolysis of allantoate in *S.allantoicus* was identical either with the allantoicase or allantoate amidohydrolase system. This was most important because VALENTINE *et al.* (1962) stated that *S.allantoicus* contained allantoicase, while VOGELS (1963, 1966) demonstrated another enzyme, allantoate amidohydrolase, to be responsible for allantoate breakdown.

The degradation of allantoate and the formation of the reaction products were followed as a function of the incubation time (Fig.6). A decrease of the total amount of glyoxylate and its acid-labile derivatives occurred (curve 1). This was measured by using method B of the differential glyoxylate analysis, in which the sum of this derivatives (allantoate, ureidoglycine, ureidoglycolate) and glyoxylate was determined. The maximal amounts of ammonia (curve 5) and glyoxylate (curve 4) formed were less than could be expected from the amount of allantoate initially present. After 60 min of incubation the sum of the amounts of ureidoglycolate and glyoxylate (curve 2) decreased and the decrease was similar to that observed for the sum of glyoxylate and its acid-labile derivatives (curve 1). This disappearance of glyoxylate and its acid-labile derivatives will be discussed later (4.3). It appeared that two moles of ammonia (curve 5) were formed per one mole of ureidoglycolate and glyoxylate produced (curve 2). From Scheme 2 (1.1.2) it followed that ammonia could be produced either by hydrolysis of urea or direct from allantoate. The first pathway of ammonia production was not operative in crude cell-free extract of *S.allantoicus*, since no ammonia was formed from urea under the conditions used in Fig.6. This experiment confirmed earlier results that no urease was present in *S.allantoicus* (VOGELS, 1963).

Similar results were obtained with purified enzyme preparations. Two moles of ammonia were formed together with one mole of ureidoglycolate and glyoxylate (Fig.7). Incubation of urea under the same conditions as given in Fig.7 did not result in ammonia production. From these results it appeared that the production of ammonia from allantoate by the enzyme from *S.allantoicus* was not catalyzed by allantoicase. Another

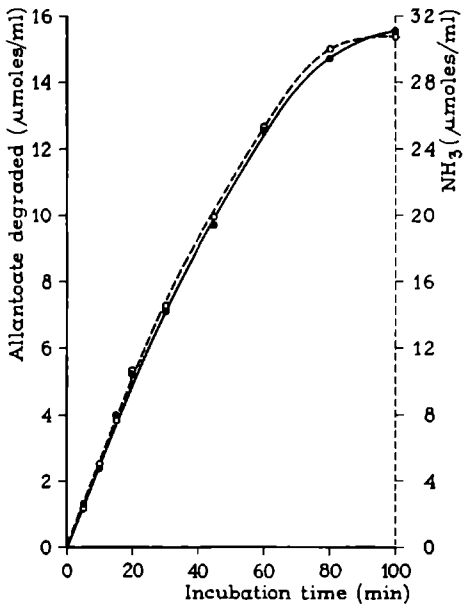


Figure 7

Degradation of allantoate by purified allantoate amidohydrolase from *S.allantoicus*. Enzyme was activated (cf. Chapter 6) by mixing 0.2 ml (12 μg protein and 34 mμmoles EDTA in 0.05 M Tris-HCl buffer, pH 7.5) with 0.6 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.95). After 30 min at 30° 2 ml buffered substrate solution were added. The final incubation mixture contained, per ml, 19.3 μmoles sodium allantoate, 0.12 μmole $MnSO_4$, 94 μmoles diethanolamine-HCl buffer (pH 8.8) and 4.1 μg protein. Incubation was performed at 30°. Curves represent the sum of ureidoglycolate and glyoxylate formed (•) and the amount of ammonia produced (o).

argument in favor of an enzyme system distinct from allantoicase was brought about by the observation that the purified enzyme preparation did not produce any allantoate from ureidoglycolate in the presence of a four-fold excess of urea. Under these conditions an enzyme preparation which contained allantoicase would have produced allantoate, since the reaction catalyzed by allantoicase was reversible (TRIJBELS, 1967). Therefore, in *S.allantoicus* an enzyme system is present which is distinct from allantoicase and will henceforth be called allantoate amidohydrolase (VOGELS, 1963, 1966). Ureidoglycine was supposed to be an intermediate in this reaction but it was not known whether this

compound was hydrolyzed by the same enzyme or another one or that it was cleaved by a non-enzymic reaction. Furthermore it was not known whether allantoate amidohydrolase produced carbon dioxide and ammonia direct or *via* carbamylic acid which in turn was converted to carbon dioxide and ammonia.

4.2 THE PRODUCTION OF (-)-UREIDOGLYCOLATE FROM ALLANTOATE

To be sure that ureidoglycolate indeed was an intermediate during allantoate hydrolysis in *S.allantoicus* several identification reactions were performed. It had already been mentioned (4.1) that during allantoate degradation a product was formed, which reacted positively in method D of the differential glyoxylate analysis as did ureidoglycolate itself. Thin-layer chromatography on cellulose (solvent system pyridine-acetic acid-water, 174 : 1 : 88, by vol.) of an aliquot of an incubation mixture and comparison with the reference compound (R_f = 0.60; spray: Ehrlich's reagent) revealed that ureidoglycolate was produced during allantoate hydrolysis. The same was proven by paper electrophoresis of an aliquot of an incubation mixture: under the experimental conditions (pyridine-acetic acid-water (10 : 1 : 8.9, by vol.; pH 6.5) for 75 min, at 1500V, 80mA, and -6°) allantoate and ureidoglycolate migrated 7.0 and 9.2 cm to the anodic side.

Since allantoate is a symmetric molecule and ureidoglycolate contains an asymmetric carbon atom, it was conceivable that during allantoate degradation only one optical isomer of ureidoglycolate should be formed. Therefore, the change of optical rotation was studied (Fig.8) in an incubation mixture of allantoate and a purified enzyme preparation (specific activity 77). GSH, a cofactor of the enzyme (5.5), was omitted because this substance itself was optically active. The degradation of allantoate was accompanied by an increase of left rotation, which paralleled the amount of ureidoglycolate (curve 1) formed. The optical activity was totally accounted for by ureidoglycolate, since calculation from the amount of ureidoglycolate formed and the observed optical rotation yielded an $[\alpha]_D^{30} = -10.5^\circ \pm 1^\circ$. This specific

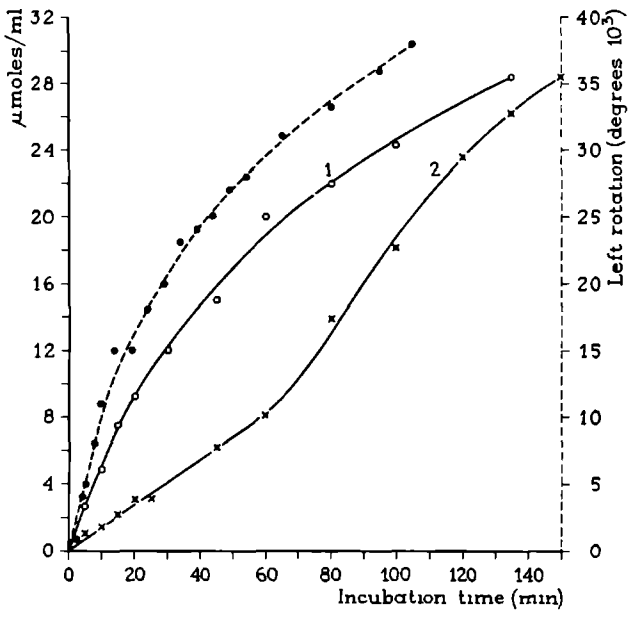


Figure 8

Formation of (-)-ureidoglycolate during allantoate hydrolysis. An enzyme preparation containing 24 µg protein and 34 mµmoles EDTA was activated as described in Fig.7. Activity was tested at 30° by the addition of 2 ml substrate solution. The assay mixture contained, per ml, 100 µmoles sodium allantoate, 0.11 µmole $MnSO_4$, 96 µmoles diethanolamine-HCl buffer (pH 8.8) and 8.5 µg protein. Optical rotation was followed in a Perkin-Elmer polarimeter with a sodium vapor lamp (dashed line). The amounts of ureidoglycolate (1) and glyoxylate (2) were determined by differential glyoxylate analysis.

optical rotation was in good agreement with that determined for (-)-ureidoglycolate, *viz.* $-10^{\circ} \pm 1^{\circ}$ (TRIJBELS and VOGELS, 1966b; TRIJBELS, 1967).

From Fig.8 it appeared that (-)-ureidoglycolate was formed during breakdown of allantoate by allantoate amidohydrolase. Therefore, it was investigated whether this optical isomer was broken down further by the ureidoglycolase present in *S.allantoicus*. Incubation of racemic ureidoglycolate with a partially purified ureidoglycolase preparation (specific activity 2.58) of this organism resulted in a change of optical

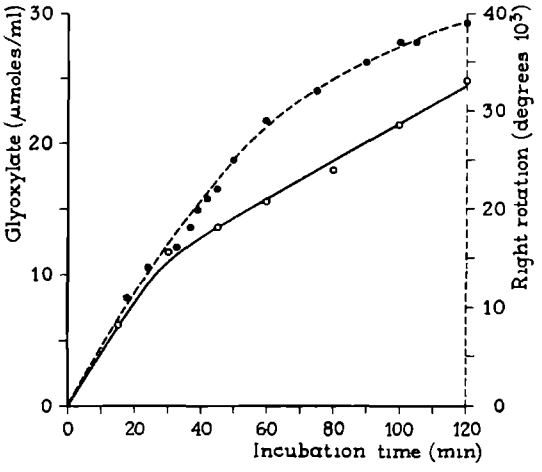


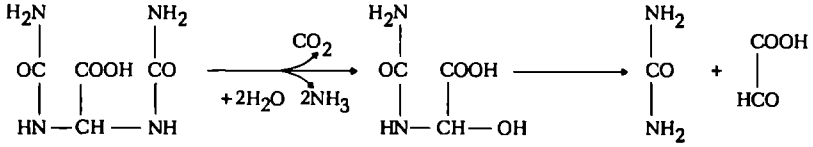
Figure 9

Degradation of (-)-ureidoglycolate by ureidoglycolase from *S.allantoicus*. The incubation mixture at 30° contained, per ml, 80 μmoles racemic sodium ureidoglycolate, 0.13 μmole $MnSO_4$, 35.4 μmoles Tris-HCl buffer, 38.2 μmoles diethanolamine-HCl buffer, 0.12 μmole EDTA and 0.16 mg protein. Final pH was 8.2. Optical rotation was measured in a Perkin-Elmer polarimeter with a sodium vapor lamp. Ureidoglycolate degradation was followed by measurement of glyoxylate production. The values were corrected for non-enzymic cleavage of ureidoglycolate.

rotation in the right direction (Fig.9). For the $[\alpha]_D^{30}$ of (+)-ureidoglycolate a value of $+10.5^\circ \pm 1^\circ$ was calculated, which was in agreement with the value calculated from Fig.8 and that reported by TRIJBELS (1967). Besides glyoxylate also urea was formed during this reaction. This was demonstrated by measurement of the amount of ammonia formed from ureidoglycolate in the presence and absence of urease. It appeared that one mole of glyoxylate and one mole of urea were formed simultaneously from one mole of ureidoglycolate. The identity of the urea formed was further confirmed by thin-layer chromatography performed under the same conditions as described above for ureidoglycolate. Also paper electrophoresis under the above-mentioned conditions revealed urea formation during ureidoglycolate hydrolysis : urea migrated 0.5 cm to the cathode.

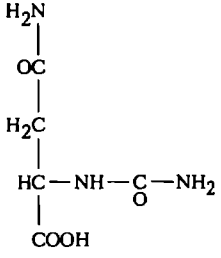
From the above-mentioned experiments it followed that the hydro-

lysis of allantoate in *S. allantoicus* proceeds along the following reaction sequence:



ALLANTOIC ACID (-)-UREIDOGLYCOLIC ACID UREA GLYOXYLIC ACID

On the basis of degradation of N-carbamoyl-L-asparagine (5.7) it was possible to denote to (-)-ureidoglycolate the L-configuration.

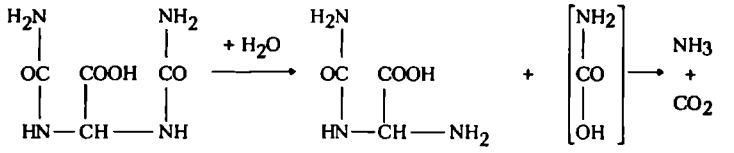


N-CARBAMOYL-L-ASPARAGINE

4.3 THE REACTION CATALYZED BY ALLANTOATE AMIDOHYDROLASE

The enzymic cleavage of allantoate results in the production of one mole of carbon dioxide, one mole of ureidoglycolate and two moles of ammonia. It is unlikely that this cleavage is a one-step reaction. VOGELS (1963) therefore postulated ureidoglycine as an intermediate (Scheme 2, 1.1.2) but so far this substance could neither be demonstrated nor synthesized. Together with ureidoglycine, then, a carbamyl group is cleaved from allantoate. Since in most cases our incubation mixtures contained phosphate which was used in the activation procedure (Chapter 6) the possibility existed that the carbamyl group would be split off phosphorolytically. However, the presence of carbamoyl phosphate as an intermediate was not probable for the following reason: in the

presence of ornithine no citrulline was formed during allantoate degradation, although crude extracts contained ornithine transcarbamoylase (carbamoyl phosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3) activity. We were unable to detect carbamylic acid as an intermediate according to the method of SUMNER (1951). The carbamyl group, if released from the allantoate molecule, is probably cleaved immediately into ammonia and carbon dioxide:



ALLANTOIC ACID UREIDOGLYCINE CARBAMYLIC ACID

4.3.1 *Production of glyoxylate during allantoate degradation*

During allantoate breakdown besides ureidoglycolate also glyoxylate was formed. For crude extracts this was understandable, since ureidoglycolase was present. Glyoxylate formation was observed also with purified allantoate amidohydrolase preparations (Fig.10). The enzyme preparation was not contaminated with ureidoglycolase since the degradation of ureidoglycolate proceeded at the same rate both in the presence and in the absence of the enzyme (curves 1 and 2). Therefore, this degradation was due to non-enzymic hydrolysis of ureidoglycolate under these conditions. With allantoate as substrate a much larger amount of glyoxylate was produced (curve 6). Therefore, it seemed likely that there was another route for glyoxylate production, which did not proceed *via* ureidoglycolate. The non-enzymic hydrolysis of ureidoglycolate formed would result in a glyoxylate production shown in curve 7, which was calculated from the amount of ureidoglycolate present at the different time intervals (curve 5) and the rate of non-enzymic hydrolysis of ureidoglycolate (curve 1). Under the experimental conditions used an amount between 30 % to 50 % of the allantoate was converted to glyoxylate and only about 5 % could have been formed *via* ureidoglycolate. It is evident that in addition to glyoxylate formation *via* ureidoglycolate still another route for the production of glyoxylate must exist.

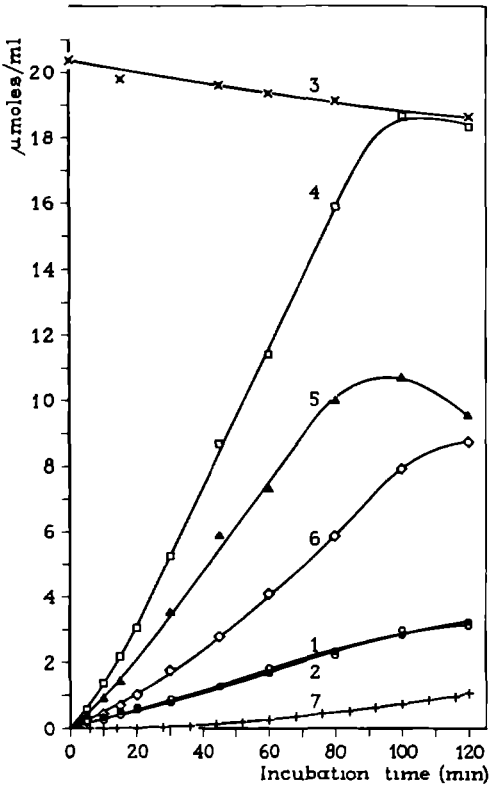


Figure 10

Formation of glyoxylate from allantoate or ureidoglycolate by purified allantoate amidohydrolase. Purified enzyme (6 µg protein) was activated as given in Fig.7. Incubation was started by the addition of substrate. The final incubation mixtures contained, per ml, 20.4 µmoles sodium allantoate (curves 3-6) or 21 µmoles sodium ureidoglycolate (curves 1 and 2), 4.27 µmoles GSH, 0.11 µmole $MnSO_4$, 93 µmoles diethanolamine-HCl buffer (pH 8.8) and 2.1 µg of the activated enzyme. Curve 1 non-enzymic decomposition of ureidoglycolate, curve 2 decomposition of ureidoglycolate in the presence of the enzyme, curve 3 disappearance of total acid-labile glyoxylate derivatives and glyoxylate itself, curve 4 the amount of ureidoglycolate and glyoxylate formed from allantoate, curve 5: ureidoglycolate formed from allantoate, curve 6 glyoxylate formed from allantoate and curve 7: glyoxylate formation from ureidoglycolate formed from allantoate (see text).

4.3.1.1 Effect of enzyme and substrate concentration on glyoxylate formation

To obtain more information on the formation of glyoxylate during degradation of allantoate the effect of enzyme and substrate concentration on the glyoxylate production was studied. In Fig.11 it was shown that the rate of allantoate degradation was proportional to the enzyme concentration (curves 1-3). However, it appeared that the less enzyme was present the more glyoxylate was formed at the end of the incubation period (curves 1a-3a), although the initial rate of glyoxylate formation was higher when more enzyme was present. When allantoate was degraded completely about 2.3, 3.6 and 7 μmoles of glyoxylate per 20 μmoles of allantoate were formed by 8.6, 4.3 and 2.15 μg of protein, respectively.

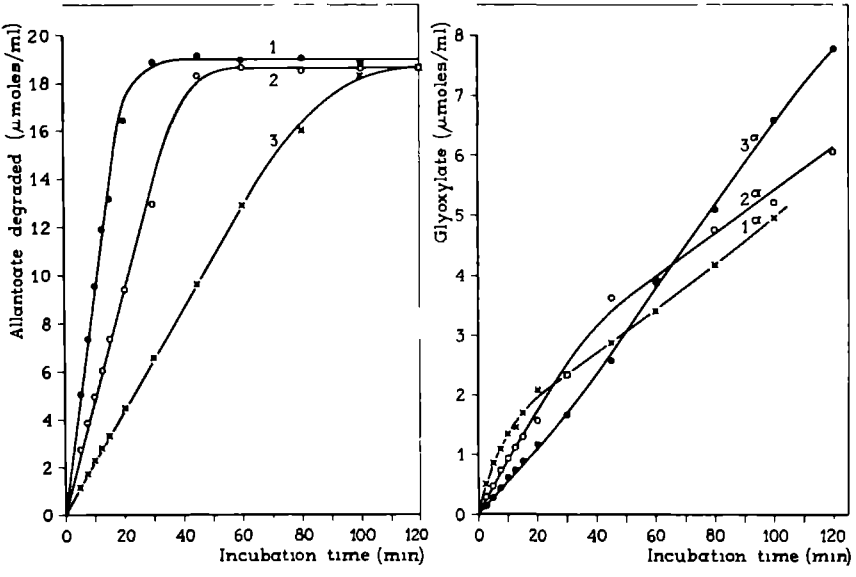


Figure 11

Degradation of allantoate with concomitant glyoxylate formation by allantoate amidohydrolase in the presence of varying amounts of enzyme. Enzyme was activated as given in Fig.7. Incubation was at 30°. The incubation mixtures contained, per ml, 20 μmoles sodium allantoate, 4.23 μmoles GSH, 0.11 μmole $MnSO_4$, 93 μmoles diethanolamine-HCl buffer (pH 8.8) and activated enzyme: 8.6 μg (1), 4.3 μg (2) and 2.15 μg (3) protein, respectively. Curves 1-3 represent allantoate degraded and curves 1a-3a the amount of glyoxylate formed.

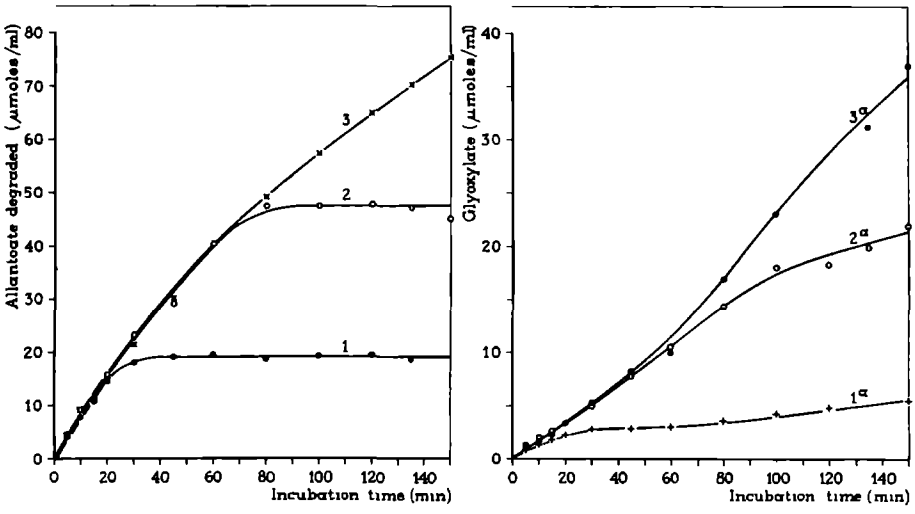


Figure 12

Degradation of allantoate with concomitant glyoxylate formation by allantoate amidohydrolase in the presence of varying amounts of substrate. Enzyme was activated as given in Fig.7. The incubation mixtures at 30° contained, per ml, sodium allantoate (20 (1 and 1a), 50 (2 and 2a) or 100 (3 and 3a) µmoles), 0.11 µmole $MnSO_4$, 4.23 µmoles GSH, 93 µmoles diethanolamine-HCl buffer (pH 8.8) and 8.6 µg activated enzyme.

The influence of variation of the substrate concentration on the rate of allantoate degradation and glyoxylate formation is shown in Fig.12. The rate of degradation was independent on the initial allantoate concentration (5.3) and the amount of glyoxylate formed was higher when the initial substrate concentration was higher. After a complete allantoate degradation 12.5 % (1), 36 % (2) and more than 50 % (3) of the allantoate were converted to glyoxylate (Fig.12, curves 1, 2 and 3, respectively). In both experiments (Figs. 11 and 12) there was a change of the rate of glyoxylate formation at the moment that allantoate was fully degraded. This could be understood, since at this point the glyoxylate formation will only proceed *via* ureidoglycolate by non-enzymic hydrolysis. The rate of glyoxylate formation after complete breakdown of allantoate indeed equalled the rate of non-enzymic decomposition of ureidoglycolate.

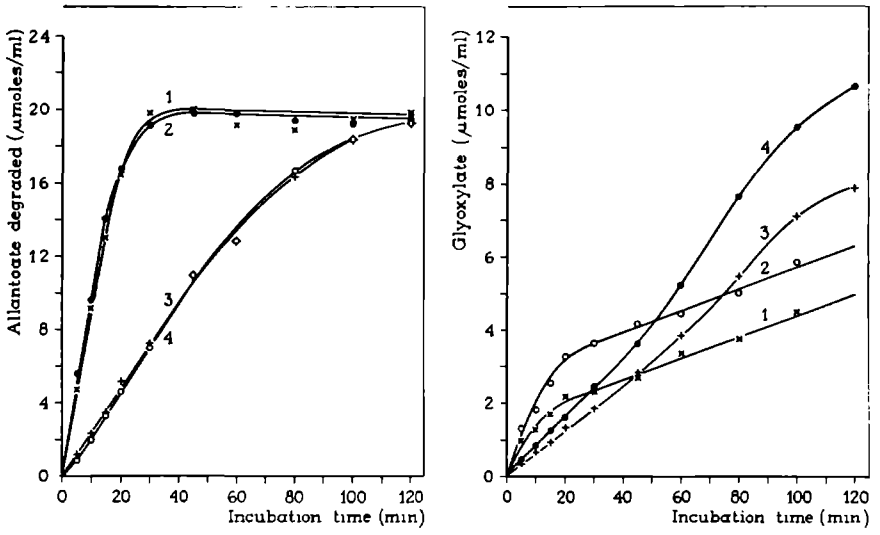


Figure 13

Influence of glyoxylate on allantoate hydrolysis and glyoxylate formation by allantoate amidohydrolase. The incubation mixtures at 30° contained, per ml, 20 μmoles sodium allantoate, 4.23 μmoles GSH, 0.11 μmole $MnSO_4$, 93 μmoles diethanolamine-HCl buffer (pH 8.8), 5 μmoles sodium glyoxylate (curves 2, 2a, 4 and 4a) and 24 μg (curves 1, 1a, 2 and 2a) or 6 μg (curves 3, 3a, 4 and 4a) activated purified allantoate amidohydrolase, respectively. Activation was performed as described in Fig.7. Curves 2, 2a, 4 and 4a were corrected for the extra added sodium glyoxylate.

4.3.1.2 Effect of some reaction products on glyoxylate formation

Urea (5 μmoles/ml), racemic ureidoglycolate (10 μmoles/ml) or carbamoyl phosphate (10 μmoles/ml), added to a test mixture as described in the legend of Fig.11, did not influence the rate of the reaction catalyzed by allantoate amidohydrolase; allantoate degradation and glyoxylate formation proceeded with the same rate as observed in the absence of these additions. However, the addition of glyoxylate (5 μmoles/ml) at the start of the incubation period had a marked effect on the amount of glyoxylate formed from allantoate, whereas the rate of allantoate disappearance was not influenced (Fig.13).

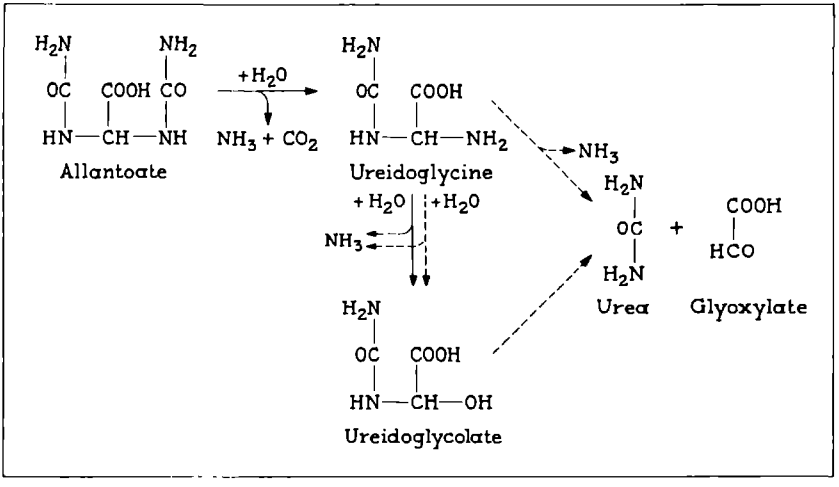
After degradation of allantoate was completed 2.4 and 3.6 μmoles of glyoxylate were formed from allantoate by 24 μg of enzyme protein in the absence and presence of glyoxylate, respectively. When 6 μg

of enzyme protein were present the glyoxylate formed amounted to 7.9 and 10.6 μmoles, respectively.

In all these experiments GSH was present. This substance could possibly interfere with the effects described, since it is known that under certain conditions glyoxylate can form a condensation product with GSH or other reducing substances (RAO and RAMAKRISHNAN, 1962). Repeating the experiments of Figs. 11-13 and omitting GSH from the assay mixtures we obtained similar results. Furthermore on incubation of 25 μmoles of glyoxylate with 5 μmoles of GSH, which was about the amount normally present during incubation, no glyoxylate had disappeared after 2 h under the experimental conditions of Figs. 11-13. The apparently stimulating effect of glyoxylate on its own production cannot be explained by focusing the attention on the non-enzymic hydrolysis of ureidoglycolate. This reaction would be more likely reversed since it was an equilibrium reaction.

4.3.2 *Conclusions on glyoxylate formation*

A tentative explanation of the phenomena observed is proposed in Scheme 4.



Scheme 4

A hypothetic scheme of allantoate degradation in *Streptococcus allantoicus:* enzymic (full lines) and non-enzymic (dashed lines) reactions.

Since allantoate amidohydrolase was saturated at very low concentrations of substrate (5.3) the rate of allantoate degradation to ureidoglycine was, under the conditions used, independent on the substrate concentration. Thus, the formation of ureidoglycine proceeded always at the same rate. At higher substrate concentrations more glyoxylate was formed but the initial rate of formation was the same (Fig.12). A higher amount of glyoxylate formed can be a result of three effects (Scheme 4): 1) stimulation of the non-enzymic decomposition of ureidoglycolate or 2) a stimulation of the direct conversion of ureidoglycine to glyoxylate or 3) inhibition by allantoate of the conversion of ureidoglycine to ureidoglycolate; consequently, more ureidoglycine accumulates and more glyoxylate is formed, since ureidoglycine appeared to be more unstable than ureidoglycolate. The first possibility was excluded by measuring the non-enzymic hydrolysis of ureidoglycolate in the presence and absence of allantoate and/or ammonia. No difference in the rate of hydrolysis was observed. The second possibility, *viz.* stimulation of the non-enzymic conversion of ureidoglycine to glyoxylate by allantoate, is improbable, since the initial rate of glyoxylate formation was independent on the allantoate concentration. Consequently, allantoate must inhibit the conversion of ureidoglycine to ureidoglycolate. Presumably, also this reaction can be catalyzed by the enzyme allantoate amidohydrolase.

The postulation that ureidoglycine was an intermediate during allantoate hydrolysis (VOGELS, 1963; 4.3) is strengthened by the observations on glyoxylate formation from allantoate. Further evidence will be given in the next section.

4.4 FORMATION OF GLYCINE DURING ALLANTOATE DEGRADATION

On incubation of crude cell-free extracts with allantoate glycine was formed. This compound could be identified by thin-layer chromatography (solvent system phenol-water, 4 : 1 (w/v):R_f = 0.39; pyridine-acetic acid-water, 174 : 1 : 88, by vol.: R_f = 0.42; n-butanol-acetic acid-water, 12 : 3 : 5, by vol.: R_f = 0.20). VOGELS (1963) already had shown that

allantoate disappearance resulted in formation of glycine. This substance was not formed direct from ureidoglycolate or glyoxylate, since incubation of crude enzyme preparations with these degradation products of allantoate did not yield glycine, either in the presence or absence of ammonia. Glycine is apparently formed from a substance that preceeds ureidoglycolate in the degradation pathway, *viz.* allantoate or more likely ureidoglycine.

Glycine production was also observed when allantoate was incubated with purified allantoate amidohydrolase. In previously mentioned experiments (Fig.10, curve 1) a small decrease of the amount of glyoxylate and its acid-labile derivatives occurred. This decrease could be due to a transamination reaction between ureidoglycine and glyoxylate as supposed by VOGELS (1963):

UREIDOGLYCINE GLYOXYLIC ACID OXALURIC ACID GLYCINE

If this reaction indeed occurs, then the amount of glycine formed should be equal to half of the amount of acid-labile glyoxylate derivatives disappeared. In Fig.14 the relationship between this disappearance and glycine formation is shown. Extra glyoxylate was added in this experiment, since VOGELS (1963) demonstrated that in the presence of glyoxylate more glycine was formed from allantoate than in the absence of added glyoxylate. Allantoate was degraded completely after about 100 min of incubation; at this time glycine production almost stopped. It was evident that both curves of the figure were sigmoidal. This could be expected on the basis of the reaction given above, because one of the substances that took part in the transamination reaction, *viz.* ureidoglycine, must have been produced from allantoate. At the end of the incubation period 0.6 µmole of glycine was formed, but 2.2 µmoles of acid-labile glyoxylate derivatives had disappeared. This amount of glycine was less than could be expected on the basis of

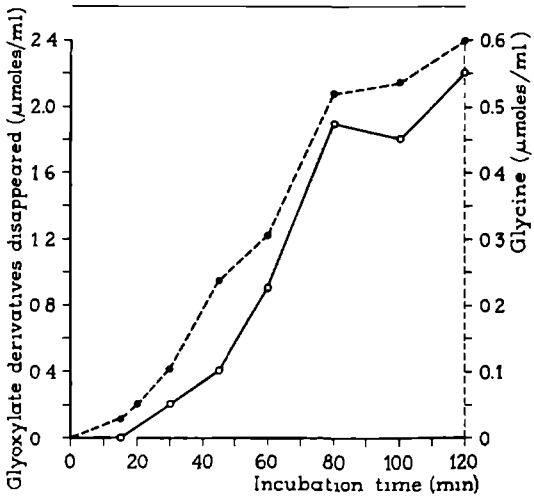


Figure 14

Formation of glycine from allantoate by purified allantoate amidohydrolase. 0.4 ml enzyme solution (36 μg protein and 68 mμmoles EDTA in 0.05 M Tris-HCl buffer, pH 7.5) was treated for 30 min at 30° with 1.2 ml 0.1 M phosphate buffer (pH 5.95) (cf. Chapter 6). Incubation was started by the addition of 4.2 ml substrate solution. The final incubation mixture at 30° contained, per ml, 18.6 μmoles sodium allantoate, 10 μmoles sodium glyoxylate, 0.26 μmole $MnSO_4$, 104 μmoles veronal-$NaHCO_3$ buffer (pH 9.3) and 6.2 μg activated enzyme. Final pH was 8.8. Samples were measured for glyoxylate and its acid-labile derivatives (full line) and glycine (dashed line).

transamination: about 50-60 % of the acid-labile glyoxylate derivatives disappeared was accounted for by glycine. Therefore, the possibility was tested whether glyoxylate was broken down non-enzymically. On incubation for 2 h of 26 μmoles of glyoxylate with 26 μmoles of NH_4Cl at pH 8.8 a decrease (about 10 %) of the amount of glyoxylate was observed. In the absence of NH_4^+ ions no disappearance of glyoxylate occurred. The amount of NH_4^+ remained constant; probably this ion acted as a catalyst in a non-enzymic conversion of glyoxylate. This would implicate that under the conditions of Fig.14 a disappearance of at most 2.8 μmoles of glyoxylate could be expected as a result of this reaction. A disappearance of about 1 μmole of glyoxylate *plus* its acid-labile derivatives could not be explained on the basis of glycine production. The apparently too high disappearance

was most likely due to the NH_4^+-catalyzed conversion of glyoxylate.

The question arose whether the enzyme ureidoglycine aminotransferase (VOGELS, 1963) existed or ureidoglycine was converted to glycine by a non-enzymic transamination with glyoxylate (NAKADA and WEINHOUSE, 1953). METZLER *et al.* (1954) and FLEMING and CROSBIE (1960) observed that such a non-enzymic transamination with glyoxylate could be promoted by the addition of Cu^{2+} ions. Therefore, we tested the effect of 5 x 10^{-4}M $CuSO_4$ on the production of glycine from allantoate. An enhancement of glycine formation was demonstrated by thin-layer chromatography (solvent system pyridine-acetic acid-water, 174 : 1 : 88, by vol.: R_f = 0.42). Addition of pyruvic acid (10 μmoles/ml) at the start of the incubation period did not markedly influence the glycine production. Alanine production in the presence of pyruvate was not observed. This was in accordance with the results of FLEMING and CROSBIE (1960), who reported that pyruvate was much less effective in non-enzymic transamination reactions as compared to glyoxylate.

In order to test the reversibility of the transamination reaction a mixture of oxalurate and glycine was incubated under the conditions of Fig.14. No appreciable formation of glyoxylate and its acid-labile derivatives occurred. Ureidoglycine formation was also not observed on incubation of ureidoglycolate with NH_4^+ under similar conditions. This was followed by measuring glyoxylate production: if ureidoglycine was formed one could expect a greater production of glyoxylate because of the direct non-enzymic hydrolysis of this substance to urea, ammonia and glyoxylate (Scheme 4).

4.4.1 *Non-enzymic degradation of 5-aminohydantoin*

Further evidence that ureidoglycine could be transformed non-enzymically into glycine was obtained from studies with 5-aminohydantoin. Treatment of this substance for 2 min at 100° with 0.1 N NaOH (pH = 13) resulted in formation of glycine, which could be demonstrated by thin-layer chromatography. A similar treatment with 0.1 N HCl (pH = 1) for 2 min at 100° did not result in glycine formation. 5-Aminohydantoin was stable at acidic pH values. When 5-aminohydantoin was incubated at 30° at a slightly alkaline pH also glycine was formed; the

Table 8

Formation of glycine and some other products from 5-aminohydantoin in the presence or absence of glyoxylate at 30° and pH 8.8

The incubation mixtures contained, per ml, 23.3 μmoles 5-aminohydantoin or 23.3 μmoles 5-aminohydantoin + 28.2 μmoles sodium glyoxylate or 28.2 μmoles sodium glyoxylate, and 150 μmoles veronal-$NaHCO_3$ buffer (pH 8.8). 5-Aminohydantoin was measured according to method A of the differential glyoxylate analysis. The notation - in this table stands for: amounts below detection.

Products	5-Aminohydantoin (μmoles/ml)		5-Aminohydantoin +glyoxylate (μmoles/ml)		Glyoxylate (μmoles/ml)	
	0 min	120 min	0 min	120 min	0 min	120 min
5-Aminohydantoin	23.3	12.6	22.5	0.4	-	-
Ureidoglycolate	1.76	0.54	-	0.27	-	-
Glyoxylate	-	0.74	28.4	0.34	28.2	28.3
Glycine	-	3.55	2.82	8.4	-	-
Urea	-	8.9	-	8.85	-	-
Ammonia	-	6.2	-	11.5	-	-

presence of glyoxylate enhanced this production considerably (Table 8). In the incubation mixture containing both 5-aminohydantoin and glyoxylate most of these substances disappeared within 2 h of incubation. About 30 % were converted to glycine. It is evident from the amounts of products given in Table 8 that a large part of 5-aminohydantoin was converted into unknown products.

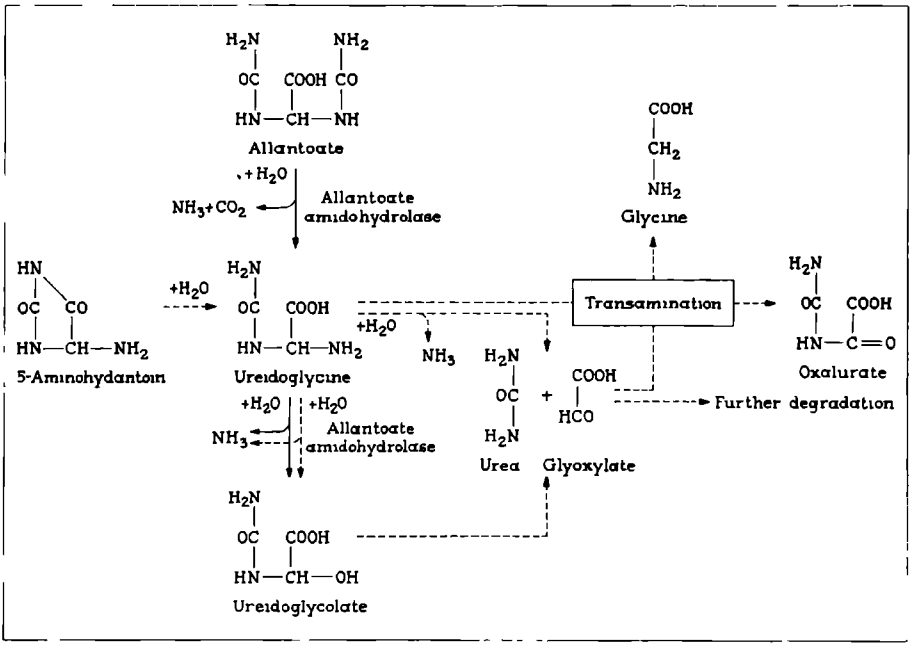
4.5 EVIDENCE FOR UREIDOGLYCINE AS AN INTERMEDIATE DURING ALLANTOATE HYDROLYSIS

In the preceding paragraphs (4.3 and 4.4) evidence for ureidoglycine as intermediate was obtained. Production of ammonia from allantoate already indicated (VOGELS, 1963) that this substance was an intermediate. The formation of glyoxylate from allantoate by purified allan-

toate amidohydrolase, which contained no ureidoglycolase activity, was also an indication that ureidoglycine was an intermediate (Scheme 4). Furthermore strong evidence was obtained from glycine production (VOGELS, 1963; 4.4) and from the non-enzymic reactions of 5-aminohydantoin at alkaline pH. No direct demonstration of ureidoglycine was possible. Presumably, this substance is very labile and directly converted to glyoxylate. The main difficulty in the elucidation of the reaction mechanism of the enzyme allantoate amidohydrolase was, therefore, the instability of some of the products, *viz.* carbamylic acid, ureidoglycine and ureidoglycolate. We believe it justified to consider ureidoglycine a real intermediate on the basis of the indirect evidence obtained. Postulation of the enzyme ureidoglycine aminotransferase (VOGELS, 1963) is not necessary, since glycine production can be explained by a non-enzymic transamination. Also the enzyme ureidoglycine aminohydrolase (VOGELS, 1963) most likely does not exist. Conversion of ureidoglycine to ureidoglycolate can be explained by assuming ureidoglycine to be a substrate for allantoate amidohydrolase. Competition between allantoate and ureidoglycine can then account for the difference in the amount of glyoxylate formed in the presence of varying amounts of enzyme and of substrate (4.3.2).

4.6 DISCUSSION AND CONCLUSIONS

VALENTINE *et al.* (1962) stated allantoicase to be the enzyme responsible for allantoate breakdown in *S.allantoicus*. These authors observed that 1.1 μmoles of urea were produced from one μmole of allantoate. If allantoicase was present in *S.allantoicus* one would expect the formation of two μmoles of urea and one μmole of glyoxylate from one μmole of allantoate. A tentative explanation for the low amount of urea produced was given by proposing a phosphorolytic cleavage of urea (VALENTINE and WOLFE, 1961b). Indeed, part of the enzymes given in their scheme is operative in *S.allantoicus*. Ureidoglycolate formed non-enzymically or enzymically from glyoxylate and urea or direct from allantoate could be dehydrogenated to oxalurate which in turn was phosphorolytically cleaved to oxamate and carbamoyl phosphate (VO-



Scheme 5

Degradation of allantoate by allantoate amidohydrolase from ***Streptococcus allantoicus:*** enzymic (full lines) and non-enzymic (dashed lines) reactions.

GELS, 1960; VALENTINE and WOLFE, 1960). This reaction sequence can be responsible for part of the urea disappearance, but it is hard to believe that exactly half of the total amount of urea formed will be degraded in this way. Our data did not support this hypothesis; we believe that an enzyme distinct from allantoicase was responsible for the allantoate hydrolysis in *S. allantoicus*. This enzyme was called allantoate amidohydrolase. The following scheme for allantoate hydrolysis by allantoate amidohydrolase from *S. allantoicus* was proposed; both enzymic and non-enzymic reactions are involved (Scheme 5).

The absence of glycine formation in *A. allantoicus*, which contained all other enzymes involved in allantoate breakdown (VOGELS, 1963), seemed to contradict a non-enzymic transamination reaction. However, carboligase, a glyoxylate-converting enzyme, was about 10 times as active in *A. allantoicus* as compared to *S. allantoicus*. This would im-

plicate a very rapid disappearance of glyoxylate formed during allantoate hydrolysis in *A. allantoicus* and consequently a low steady-state level of glyoxylate will be the result. This would result in a low rate of transamination between glyoxylate and ureidoglycine.

The metabolic pathway of allantoate degradation by allantoate amidohydrolase proposed in Scheme 5 seems to give a fair explanation of the results obtained, but cannot give an answer to the question whether a similar pathway is followed *in vivo*.

CHAPTER 5

ALLANTOATE AMIDOHYDROLASE: GENERAL PROPERTIES OF THE ENZYME FROM STREPTOCOCCUS ALLANTOICUS

In the preceding Chapter the reactions involved in the degradation of allantoate are described. In this Chapter we shall be concerned with the properties of the enzyme catalyzing this reaction sequence, *viz.* allantoate amidohydrolase. However, the most remarkable property of this particular enzyme, *viz.* reversible activation and inactivation, will be discussed separately (Chapters 6 and 7).

5.1 OCCURRENCE OF THE ENZYME ALLANTOATE AMIDOHYDROLASE

The enzyme allantoate amidohydrolase has been found only in a limited number of microorganisms. Its presence was demonstrated in *S. allantoicus*, *A. allantoicus*, *E. freundii*, *E. coli*, *E. coli* var. *acidilactici* (VOGELS, 1963) and *P. acidovorans* (TRIJBELS and VOGELS, 1966a). All these bacteria, with the exception of *P. acidovorans*, required anaerobic conditions for growth on allantoin as the sole source of carbon, nitrogen and energy. Cells of *S. allantoicus* grown aerobically on glucose or pyruvate or anaerobically on glucose did not contain this enzymic activity. BARKER (1961) stated the enzyme to be adaptive. No evidence was found for the presence of allantoate amidohydrolase in a number of plants and animals.

5.2 pH OPTIMUM

The allantoate amidohydrolase activity was studied as a function of the pH of the assay mixture. From Fig.15 it can be concluded that the pH optimum was in the range 8.5-9.5. Phosphate buffers (dashed line)

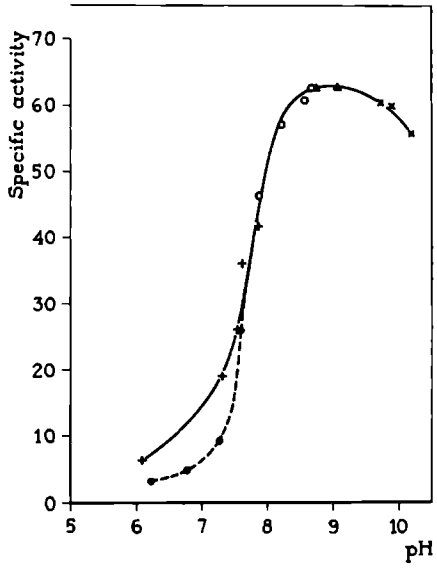


Figure 15

pH-activity curve of allantoate amidohydrolase. The experiment was performed in mixtures containing, per ml, 30 μmoles sodium allantoate, 5 μmoles GSH, 0.13 μmole $MnSO_4$, 8.9 μg purified allantoate amidohydrolase and 83 μmoles Na_2HPO_4 - KH_2PO_4 buffer (pH 6 - 7.6) (dashed line), Tris-acetic acid buffer (pH 6 - 7.9), diethanolamine-HCl buffer (pH 8.7 -9.1) or Na_2CO_3-$NaHCO_3$ buffer (pH 9.7 - 10.2). Incubation was at 30°.

exerted below pH 7.5 an inhibitory effect on the enzymic activity. This was observed for several enzymes in allantoin degradation: allantoinase (VOGELS and VAN DER DRIFT, 1966), allantoicase and ureidoglycolase (TRIJBELS, 1967). Inhibition could be partly due to binding of Mn^{2+} ions, which are cofactors for catalytic activity of the enzymes (VOGELS and VAN DER DRIFT, 1966).

The left side of the pH-activity curve, below pH 8, will be influenced by inactivation of the enzyme in the presence of Mn^{2+} ions (7.2). A similar curve was reported by VOGELS (1963). The optimal pH for the enzyme allantoate amidohydrolase was higher than those reported for several allantoicases from different sources (TRIJBELS, 1967).

5.3 REACTION RATE OF ALLANTOATE AMIDOHYDROLASE AS A FUNCTION OF THE ENZYME AND SUBSTRATE CONCENTRATION

In Fig.16 the rate of hydrolysis of allantoate is plotted as a function of the enzyme concentration. In the region tested, from 0.27 to 2.2 μg of purified enzyme protein per ml, the reaction rate was linearly proportional to the enzyme concentration.

The effect of allantoate concentration on the enzymic activity of allantoate amidohydrolase is given in Fig.17. To evaluate K_m and V_{max} the values obtained were plotted according to the method of HOFSTEE (1952, 1959). The Michaelis constant, K_m, is the negative slope of the curve and the maximal velocity, V_{max}, the intercept on the vertical axis. The values calculated for K_m and V_{max} were 1.18 x 10^{-3} M and 2.78 μmoles per ml, respectively. This calculated V_{max} yielded a higher value (about 20 %) than that determined from the velocity *vs.* substrate concentration curve. Therefore, also the

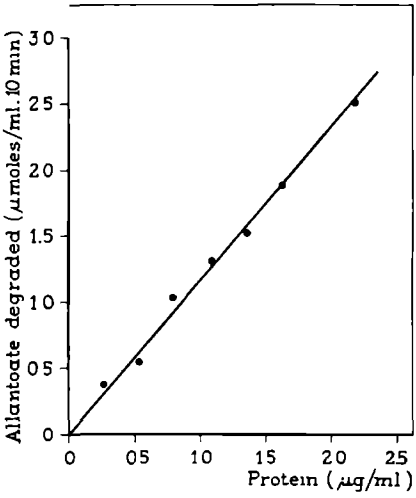


Figure 16

The rate of the allantoate amidohydrolase reaction as a function of the enzyme concentration. The incubation mixtures contained, per ml, 29.2 μmoles sodium allantoate, 4.3 μmoles GSH, 0.14 μmole $MnSO_4$, 118 μmoles diethanolamine-HCl buffer (pH 8.8) and varying amounts of purified enzyme, activated at pH 6 in the presence of phosphate and EDTA (cf. Chapter 6). Incubation was at 30°. Allantoate degraded was measured by the differential glyoxylate analysis.

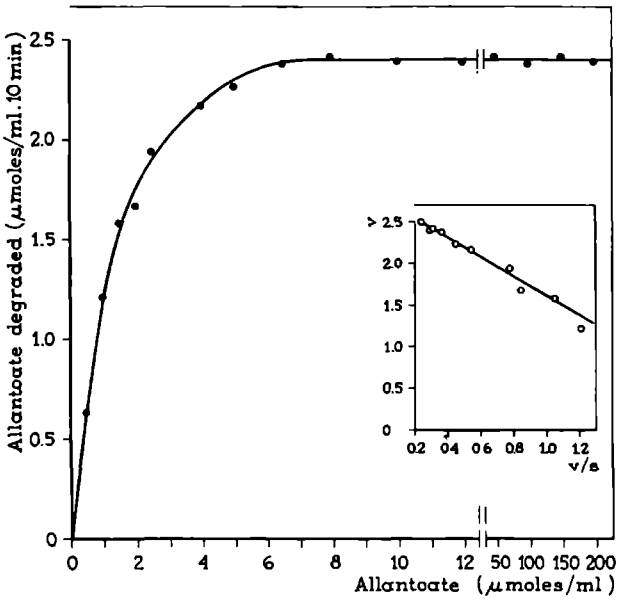


Figure 17

Rate of the allantoate amidohydrolase reaction as a function of the allantoate concentration. Incubation mixtures contained, per ml, 106 μmoles diethanolamine-HCl buffer (pH 8.8), 4.4 μmoles GSH, 0.14 μmole $MnSO_4$, 2.6 μg purified enzyme, activated at pH 6 in the presence of phosphate and EDTA (cf. Chapter 6) and sodium allantoate as indicated in the figure. Incubation was at 30°.

K_m value will be uncertain. It was a striking feature that the enzyme was saturated at a very low concentration of allantoate. This may be due to the fact that ureidoglycine can also be a substrate for the enzyme and that consequently part of the enzyme will be loaded with this compound.

5.4 TIME COURSE OF THE ENZYMIC REACTION

Hydrolysis of allantoate by the enzyme proceeded linearly with the time of incubation (cf. Chapter 4, Figs. 6 and 7).

5.5 COFACTORS OF ALLANTOATE AMIDOHYDROLASE

Dialyzed crude cell-free extracts did not degrade allantoate, neither after activation of the enzyme. Since crude extracts contained Mn^{2+} ions (cf. Chapter 7) the effect of this ion on the enzymic activity was studied. In Fig.18 the effect of the concentration of Mn^{2+} ions on the activity of purified enzyme is illustrated. It appeared that Mn^{2+} ions were essential for the catalytic action of the enzyme. Optimal activity was observed between 10^{-5} M and 5 x 10^{-4} M Mn^{2+}. In the absence of these ions no activity was observed. VOGELS (1963) obtained a similar curve but this author observed a sharp decrease of the catalytic activity of the enzyme at Mn^{2+} concentrations above 10^{-4} M. Addition of EDTA in a sufficiently high amount to the incubation

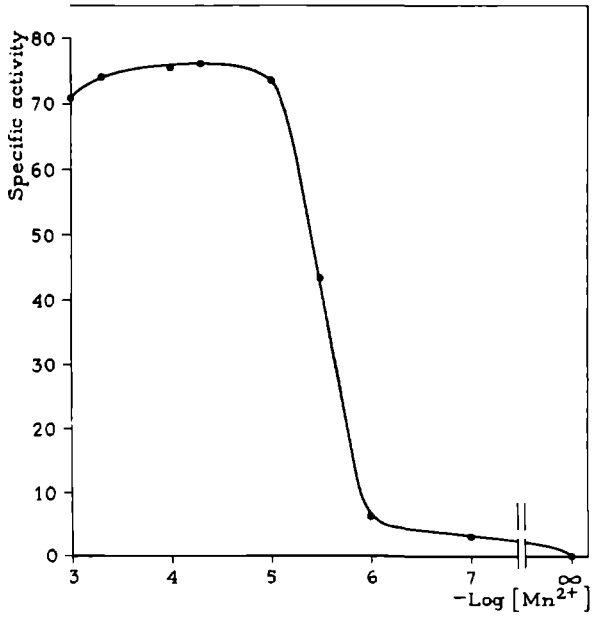


Figure 18

Effect of Mn^{2+} ions on the rate of the allantoate amidohydrolase reaction. $MnSO_4$ was tested in a concentration range from 10^{-3}M to 10^{-7}M in incubation mixtures containing, per ml, 34.4 μmoles sodium allantoate, 4.94 μmoles GSH, 3.8 mμmoles EDTA, 108 μmoles diethanolamine-HCl buffer (pH 8.8) and 0.9 μg purified allantoate amidohydrolase. Incubation was at 30°.

mixture resulted in a decrease of enzymic activity because a complex was formed between EDTA and Mn^{2+}.

A number of other bivalent cations was also tested for cofactor activity at the pH optimum. Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+} and Hg^{2+} (all 10^{-4} M) exhibited no cofactor activity but Mg^{2+}, Co^{2+}, Cd^{2+}, Ca^{2+} and Pb^{2+} (all 10^{-4} M) could partly replace Mn^{2+} (Fig. 19). VOGELS (1963) found that only Mn^{2+}, Co^{2+} and Ca^{2+} were cofactors. This contrast of results might be explained by the difference in purity of the enzyme preparations used. A similar phenomenon was observed with arginase (L-arginine amidinohydrolase, EC 3.5.3.1; BACH and

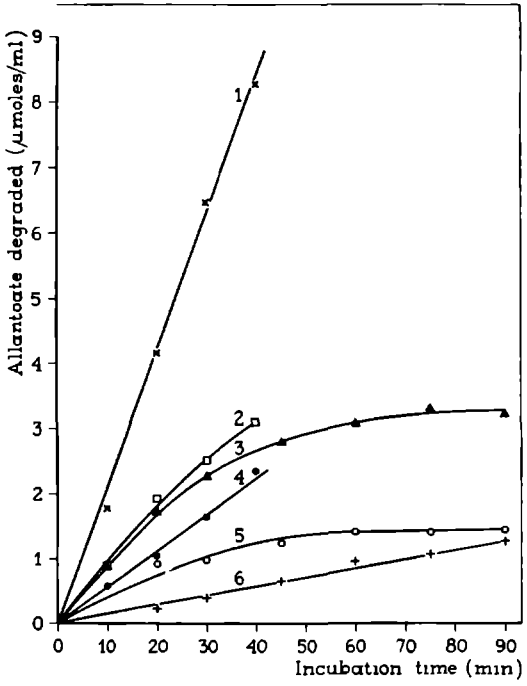


Figure 19

Influence of bivalent cations on the reaction rate of allantoate amidohydrolase. The incubation mixtures contained, per ml, 31.2 μmoles sodium allantoate, 4.7 μmoles GSH, 118 μmoles diethanolamine-HCl buffer (pH 8.8), 0.1 μmole Mn^{2+} (1), Mg^{2+} (2), Cd^{2+} (3), Co^{2+} (4), Ca^{2+} (5) or Pb^{2+} (6), and 2.1 μg purified enzyme, activated at pH 6 in the presence of phosphate and EDTA (cf. Chapter 6). Incubation was at 30^{o}.

WHITEHOUSE, 1954), phosphorylase kinase (ATP: phosphorylase phosphotransferase, EC 2.7.1.38; KREBS and FISCHER, 1956) and allantoicase (TRIJBELS, 1967).

Furthermore we investigated the effect displayed by bivalent cations (10^{-4} M) added to assay mixtures containing an optimal Mn^{2+} concentration (10^{-4} M). Only Co^{2+}, Cu^{2+} and Zn^{2+} did influence the enzymic reaction. Co^{2+}, itself a cofactor, inhibited 35 %, Cu^{2+} about 8 % and Zn^{2+} inhibited 49 %. Inhibition by Co^{2+} ions was negligible at 3 x 10^{-5}M Co^{2+} and 10^{-4} M Mn^{2+}. In the absence of any added cations no catalytic activity was obtained. This was not due to instability of the enzyme in the absence of cations (7.3), since in the presence of EDTA (up to 10^{-3} M) the enzyme was catalytically inactive too. This compound stabilized the enzyme at the optimal pH (7.3). The enzyme allantoate amidohydrolase, therefore, required essentially the presence of certain bivalent metal cations.

Sulfhydryl compounds (*e.g.* cysteine, thioglycolic acid and GSH) could act as enzyme cofactors. However, these substances were not essential but only stimulated the enzymic activity (Fig.20). Already low concen-

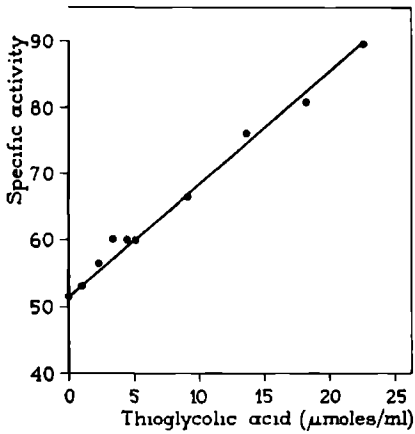


Figure 20

The activity of allantoate amidohydrolase as a function of the thioglycolic acid concentration. The experiment was performed in mixtures containing, per ml, 32.8 μmoles sodium allantoate, 0.14 μmole $MnSO_4$, 127 μmoles diethanolamine-HCl buffer (pH 8.8), varying amounts of thioglycolic acid and 2.8 μg purified allantoate amidohydrolase. Incubation was at 30°.

trations of cysteine interfered in the glyoxylate analysis (TRIJBELS, 1967). Therefore, we used GSH and thioglycolic acid which were equally effective in enzyme stimulation. In the presence of higher concentrations of these compounds (above 0.3 µmole per sample used in the analysis) the color developed in the glyoxylate analysis was too low. In our experiments routinely 4-5 µmoles of these compounds per ml incubation mixture were used to avoid this difficulty. This amounted to about 0.09-0.17 µmole of the reducing substance in the analysis sample. From the result of Fig.20 it appeared that the specific activity measured was not optimal and could still be enhanced by an increasing amount of thioglycolic acid. VOGELS (1963) found GSH to be essential for a partially purified enzyme preparation.

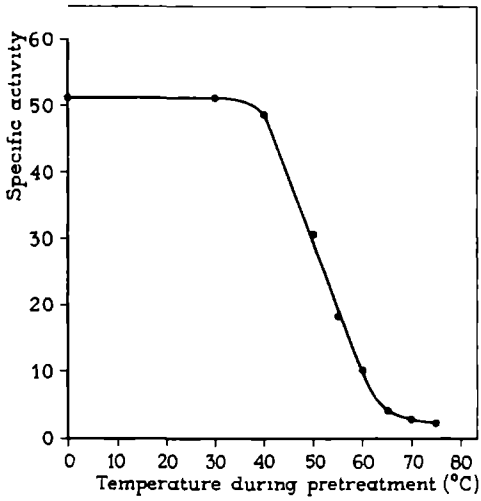


Figure 21

Influence of heat-pretreatment at pH 7.5 on the activity of allantoate amidohydrolase. Mixtures containing, per ml, 50 µmoles Tris-HCl buffer (pH 7.5), 85 mµmoles EDTA and 8.5 µg purified enzyme protein were heated for 5 min at the indicated temperatures. Incubation was started by the addition of a 0.2 ml aliquot to 2 ml substrate solution. The final incubation mixture at 30° contained, per ml, 23 µmoles sodium allantoate, 0.14 µmole $MnSO_4$, 118 µmoles diethanolamine-HCl buffer (pH 8.8) and 3.9 µg protein.

5.6 HEAT - STABILITY OF THE ENZYME

The influence of heat-pretreatment on the enzymic activity of allantoate amidohydrolase is shown in Fig.21. Above 45° a rapid decline in activity was observed. Mn^{2+} ions did not protect the enzyme during heat-treatment as was observed with several allantoicases (TRIJBELS, 1967). On the contrary, on heating the enzyme in the presence of Mn^{2+} we obtained lower specific activities, *e.g.* at 50° a specific activity of 4.5 was measured when Mn^{2+} ions were present during heat-pretreatment of the enzyme. In the absence of Mn^{2+} the specific activity was 30 (Fig.21). From the results described in Chapter 7 this large decrease of specific activity can be explained, since Mn^{2+} ions inactivated the enzyme at pH 7.5. The enzyme was not stabilized against heat-denaturation at pH 7.5 by EDTA (10^{-4} M), allantoate (0.17 M) or GSH (0.03 M). At pH 8.5 only 30 % decrease of activity took place by pretreatment for 5 min at 55° in the presence of Mn^{2+}; this was intelligible because at this pH value Mn^{2+} protected the enzyme against the decrease of activity observed in the absence of this ion (7.3.2). For this reason we did not measure the heat-stability of allantoate amidohydrolase at this pH in the absence of Mn^{2+} ions.

5.7 SUBSTRATE SPECIFICITY

Besides allantoate also 4-methylallantoate was broken down by allantoate amidohydrolase (Fig. 22). Only 50 % of this substance was degraded which indicated that only one of the optical isomers was hydrolyzed by the enzyme. The endproducts of the reaction were glyoxylate and ammonia. No urea was formed, since the amount of ammonia already present did not increase when after 2 h of incubation urease was added. Also we did not observe the formation of ureidoglycolate, since color formation in methods C and D (2.2) of the differential glyoxylate analysis was equal at every time interval tested. This would mean that methylurea and not urea was an endproduct of the reaction and that 4-methylureidoglycolate and not ureidoglycolate was an intermediate during hydrolysis of 4-methylallantoate. Therefore, the

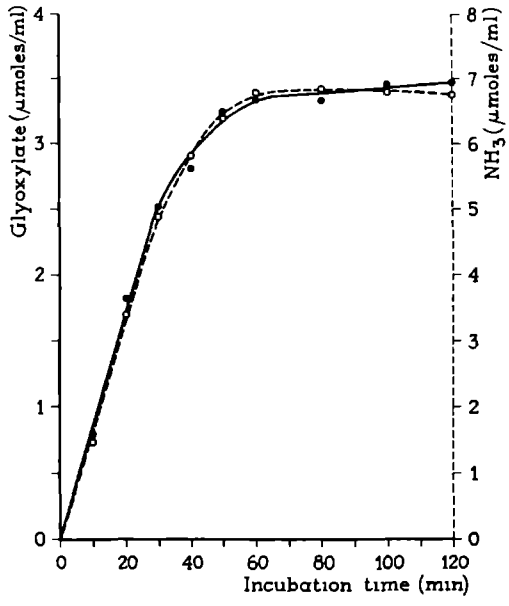
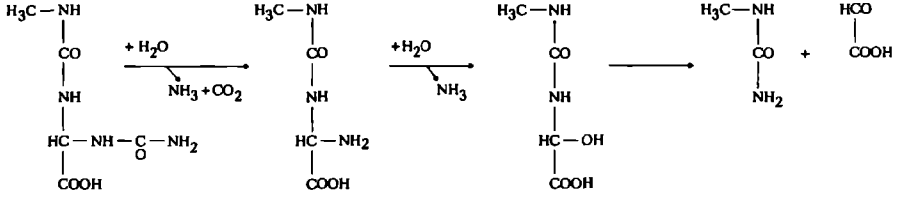


Figure 22

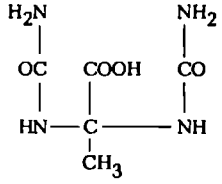
Hydrolysis of 4-methylallantoate by allantoate amidohydrolase. The assay mixture contained, per ml, 7.2 μmoles sodium 4-methylallantoate, 4.23 μmoles GSH, 0.11 μmole $MnSO_4$, 93 μmoles diethanolamine-HCl buffer (pH 8.8) and 6.3 μg purified allantoate amidohydrolase, activated at pH 6 in the presence of phosphate and EDTA (cf. Chapter 6). Incubation was at 30°. At various time intervals glyoxylate (•) and ammonia (o) were determined.

following reaction sequence was proposed:



4-METHYLALLANTOIC ACID | 4-METHYLUREIDOGLYCINE | 4-METHYLUREIDOGLYCOLIC ACID | METHYLUREA | GLYOXYLIC ACID

The other isomer of 4-methylallantoate was not degraded by the enzyme. Allantoate amidohydrolase hydrolyzed 4-methylallantoate with about 34 % of the velocity observed with allantoate as substrate.

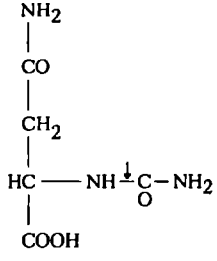


was not hydrolyzed by the enzyme.

HOMOALLANTOIC ACID

A number of N-carbamoyl derivatives of amino acids was tested as substrate because of their structural analogy with allantoate. The N-carbamoyl derivatives of glycine, L-alanine, D-alanine, L-aspartic acid, L-glutamine and D-asparagine could not substitute allantoate as substrate, but N-carbamoyl-L-asparagine was degraded by the enzyme with about 10 % of the velocity observed with allantoate. Argininosuccinic acid, hippuric acid, oxaluric acid, allantoin, 5-aminohydantoin, hydantoin, β-ureidopropionic acid, leucylnaphtylamide, L-asparagine, L-glutamine, L-arginine, L-ornithine, L-citrulline, L-lysine, glycylglycine and glycylalanine ethylester were not degraded. This was tested by measuring ammonia and/or glyoxylate production. Argininosuccinic acid, hippuric acid, glycylglycine and glycylalanine ethylester were tested for degradation by means of thin-layer chromatography after incubation.

Since N-carbamoyl-L-asparagine, and not the D-isomer, was degraded and the optical configuration of this substance was known, *viz.*:



it was probable that the enzyme allantoate amidohydrolase attacked the bond indicated by an arrow. This implicated that allantoate was broken down in the same way: from allantoate L-ureidoglycolate was then formed. In polarimetric studies the formation of (-)-ureidoglycolate was observed (4.2). Therefore, it was possible to conclude that the absolute configuration of (-)-ureidoglycolate obtained during allantoate hydrolysis was the L-form.

5.8 INHIBITION OF ALLANTOATE AMIDOHYDROLASE

In experiments on glycine formation it was observed that diethanolamine buffers interfered in glycine determination (2.2). Therefore, other buffers were substituted for diethanolamine buffer. However, it was observed that some buffer systems were inhibitory for the enzyme. Borax buffer (0.13 M) almost completely inhibited the enzymic reaction. Veronal-$NaHCO_3$ buffer (0.2 M) inhibited the enzyme about 20%; Na_2CO_3-$NaHCO_3$ buffer (0.2 M) exerted no inhibiting effect. Therefore, veronal must be inhibiting. Inhibition by borate was encountered with several enzymes and mostly was unspecific (ZITTLE, 1951). An explanation was given by BEREZIN *et al.* (1966) for inhibition of α-chymotrypsin (EC 3.4.4.5) by borate, *viz.* an effect on the unshared electron pair in the imidazole ring of histidine. Veronal was reported to act inhibitory on several enzymes, *e.g.* NAD(P)H dehydrogenase (reduced NAD(P): (acceptor) oxidoreductase, EC 1.6.99.2) and NADH oxidase. These enzymes are flavoproteins and inhibition was due to competition with substrate (CASOLA, GIUDITTA and ROCCA, 1964). The role of the prosthetic group in inhibition was not established.

No inhibition of allantoate amidohydrolase was observed in the presence of L-lysine, L-ornithine, L-arginine and L-citrulline in a ratio to substrate of 1 : 3.

Sodium bisulfite ($NaHSO_3$) was reported to decrease glycine formation from allantoate (VOGELS, 1963). This can be explained by the removal of glyoxylate formed from allantoate by $NaHSO_3$: 35 % inhibition of enzymic activity was observed in the presence of 5 mM $NaHSO_3$. Arsenite (1 mM) did not inhibit the enzyme. Since it is

known that arsenite can act on SH-groups (WEBB, 1966) most likely these groups were not essential for catalytic activity. This was further evidenced by the ineffectiveness of PCMB (10^{-4}M), both at pH 7.5 and 8.5, with and without preincubation with the enzyme for 10 min at 30°. Although the enzyme could be stimulated by sulfhydryl compounds (5.5) no inhibition by sulfhydryl-blocking agents was observed. Therefore, the role of GSH probably did not consist in stabilization of SH-groups at the active site of the enzyme molecule.

BARKER (1961) reported accumulation of allantoate during allantoin fermentation in *S. allantoicus* in the presence of sodium fluoride (0.05 M). This pointed to inhibition of an enzyme(s) operative in allantoate degradation. Since allantoate amidohydrolase was the first enzyme in this metabolic pathway, the influence of NaF on the activity of this enzyme was investigated. F^- inhibited the enzyme strongly: 3.4 x 10^{-4}M and 3.4 x 10^{-5}M yielded 84 % and 31 % inhibition, respectively. These experiments were performed in the presence of phosphate which was used in the activation procedure (cf. Chapter 6). It is known that F^- can form poorly dissociated complexes with Mg^{2+} and phosphate and thereby in-

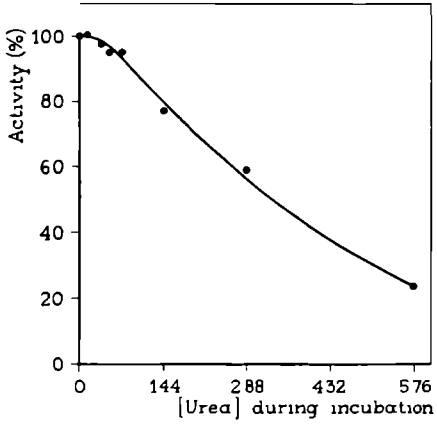


Figure 23

Effect of urea concentration in the incubation mixture on the activity of allantoate amidohydrolase. The incubation mixtures contained, per ml, 30 µmoles sodium allantoate, 5 µmoles GSH, 0.14 µmole $MnSO_4$, 112 µmoles diethanolamine-HCl buffer (pH 8.8), varying amounts of urea and 2.6 µg purified enzyme. Incubation was at 30°. The specific activity, expressed as 100 %, was 51.2.

hibit *e.g.* enolase (2-phosphate-D-glycerate hydro-lyase, EC 4.2.1.11). We tested if phosphate anions took part in F^- inhibition of allantoate amidohydrolase. In the absence of phosphate anions also a very strong decrease of enzymic activity was observed: 70% and 96 % inhibition was measured in the presence of 10^{-4}M and 10^{-3}M F^- ions, respectively. When the Mn^{2+} concentration was raised up to 10^{-3}M the same inhibition was observed as with the lower (10^{-4}M) Mn^{2+} ion concentration. Therefore, inhibition of allantoate amidohydrolase by F^- was not due to removal of Mn^{2+}, an essential cofactor (5.5), by formation of a Mn^{2+}-F^--PO_4^{3-} complex.

Urea in relatively high concentrations reduced the velocity of the reaction catalyzed by allantoate amidohydrolase. In Fig.23 the effect of the urea concentration in the incubation mixture is represented. An almost linear decrease of activity was observed when the urea molarity was increased. The effect of preincubation of the enzyme with urea on the enzymic activity is shown in Fig.24. It followed that 5.75 M urea present in both the preincubation and incubation mixture completely abolished the catalytic activity of the enzyme (curve 5). The inhibiting effect of urea during preincubation was reversible since dilution of the mixture resulted in a gradual restoration of enzymic activity (curve 4). Reversible urea denaturation is a phenomenon observed with a great number of proteins. The effect of urea is thought as a breakage of hydrogen bonds and hydrophobic linkages, but a definite explanation is not available at this moment (SCHERAGA, 1963; NÉMETHY, 1967).

A number of substances more or less related to allantoate was tested for possible inhibition of the allantoate hydrolysis. Racemic ureidoglycolate, urea (in low concentrations, <0.3 M), ammonia, homoallantoate, allantoin, hydantoin, β-ureidopropionate and the N-carbamoyl derivatives of L-glutamine, L-alanine, D-alanine and glycine did not affect the rate of the hydrolysis. 5-Aminohydantoin, N-carbamoyl-D-asparagine, N-carbamoyl-L-aspartate, N-carbamoyl-L-asparagine and hydroxyurea inhibited 12.5%, 9%, 17%, 46% and 66%, respectively, when present in the same concentration as allantoate (25 μmoles/ml). The substantial inhibition by N-carbamoyl-L-asparagine can be explained by a competition between allantoate and this compound for the

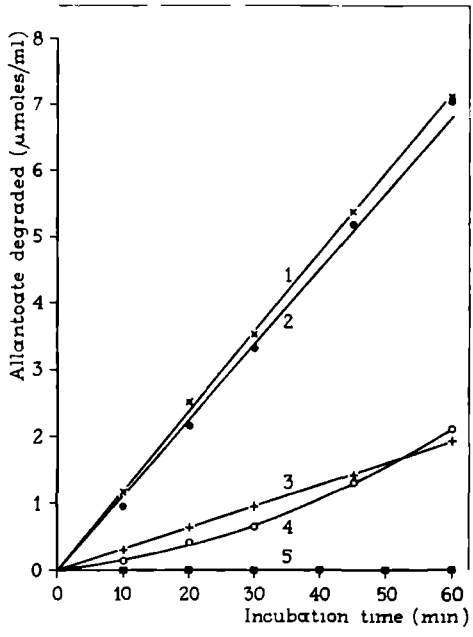


Figure 24

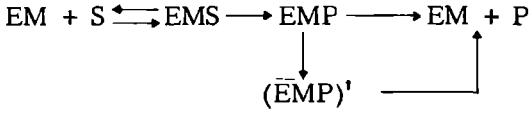
Effect of preincubation with urea on the activity of allantoate amidohydrolase. Enzyme was pretreated for 10 min at 30° with Tris-HCl-EDTA buffer, pH 7.5 (curves 1-3) or with urea (final concentration 5.75 M) in this buffer (curves 4 and 5). The assay mixture contained, per ml, 30 μmoles sodium allantoate, 5 μmoles GSH, 0.14 μmole $MnSO_4$, 112 μmoles diethanolamine-HCl buffer (pH 8.8), 2.6 μg purified enzyme protein and urea in the following concentrations: 0.26 M (1), 0.52 M (2 and 4) and 5.75 M (3 and 5). Incubation was at 30°.

enzyme, since the latter substance is also a substrate for allantoate amidohydrolase (5.7).

5.9 DISCUSSION AND CONCLUSIONS

Until now the enzyme allantoate amidohydrolase is known to occur only in five microorganisms grown on allantoin as the sole source of carbon, nitrogen and energy. The enzyme from *S. allantoicus* absolutely required bivalent cations; in the absence of these ions no activity was observed. Mn^{2+} ions were the best cofactors; several other bivalent

cations (Mg^{2+}, Cd^{2+}, Co^{2+}, Ca^{2+} and Pb^{2+}) could replace Mn^{2+} as co-factor. The stimulating effect of cofactors will be dependent on the complex stability constant and the formation rate constant. For example calcium and magnesium complexes have about the same stability constants, but the formation rate constants for Ca^{2+} are about 10^3 larger than for Mg^{2+}. Thus a Ca^{2+} - complex will dissociate much faster (10^3 times) than the corresponding Mg^{2+} - complex (EIGEN and HAMMES, 1963).



If the rearrangement to (EMP)' is necessary, then Mg^{2+} will be a better cofactor than Ca^{2+} (HAGUE and EIGEN, 1966). During allantoate hydrolysis by allantoate amidohydrolase at first ureidoglycine is formed (EMP). The conversion to ureidoglycolate (EMP)' is slow (4.3.2). This might explain the better cofactor activity of Mg^{2+} compared to Ca^{2+}.

From the bivalent cations tested only Zn^{2+} was a strong inhibitor of the enzymic reaction.

Sulfhydryl compounds acted as stimulators of the enzymic activity but were not essential. VOGELS (1963) reported GSH to be essential for purified enzyme preparations, but only stimulatory for the enzyme from crude cell-free extracts. During the present investigation with all enzyme preparations, crude or purified, an enhancement of the catalytic activity was observed but GSH was in no case an essential cofactor. Since several sulfhydryl-blocking agents, *e.g.* iodoacetate (VOGELS, 1963), PCMB, arsenite and Hg^{2+} did not influence the activity of the enzyme, it appeared that SH-groups could not be direct involved in the action of the active site of the enzyme. The role of GSH remained somewhat obscure at this moment. Several effects were displayed: stimulation of the enzyme activity at the optimal pH (5.5), stabilization at pH 8.5 (7.4) and inhibition of the activation of the enzyme at pH 6 (6.5).

The enzyme was not heat-stable as several other enzymes in allantoin metabolism.

Up to now only three substrates for allantoate amidohydrolase are known: allantoate, 4-methylallantoate and N-carbamoyl-L asparagine.

The latter compound yielded ammonia, carbon dioxide and L-asparagine. Therefore, it was possible to denote sterically the bond attacked in the substrate allantoate and to determine the absolute configuration of the ureidoglycolate formed: (-)-ureidoglycolate = L-ureidoglycolate. Consequently, (-)- and (+)- ureidoglycolase (TRIJBELS, 1967) are L- and D-ureidoglycolase.

CHAPTER 6

ACTIVATION OF ALLANTOATE AMIDOHYDROLASE

Cell-free extracts of *S.allantoicus* and of other microorganisms, which grow under anaerobic conditions on allantoin, contained only small allantoate amidohydrolase activity (VOGELS, 1966). This activity could be enhanced about 30 - 90 times by a short pretreatment at pH values below 4.5 and at 0^{o}. No removal of low- or high-molecular parts from the enzyme could be demonstrated in this activation process and no explanation could be given of the phenomenon observed (VOGELS, 1966).

This prompted us to study the phenomenon of 'acid-activation' more in detail to obtain information on the chemical alterations of this particular enzyme during this process. In most of our experiments we studied the crude or purified enzyme from *S.allantoicus*, since this organism was a good source for the enzyme and furthermore because the enzyme from this bacterium was stable for long periods.

6.1 pH-DEPENDENT ACTIVATION

6.1.1 *Acid-activation*

In Fig.25 an activation curve is shown for the enzyme from crude extracts of *S.allantoicus* and *A.allantoicus*. At the chosen pH of 3.87 and at 0^{o} the specific activity of both enzymes increased very rapidly and reached a maximal value within 1 min. The specific activity decreased rapidly in the following minutes but thereafter tended to reach a constant value which was about 40 *(S.allantoicus)* and 9 *(A.allantoicus)* times higher than the specific activity of the untreated enzymes. The activation of the enzyme was brought about in 0.04 M sodium citrate-HCl buffer (pH 3.87). Similar results were obtained when the pH

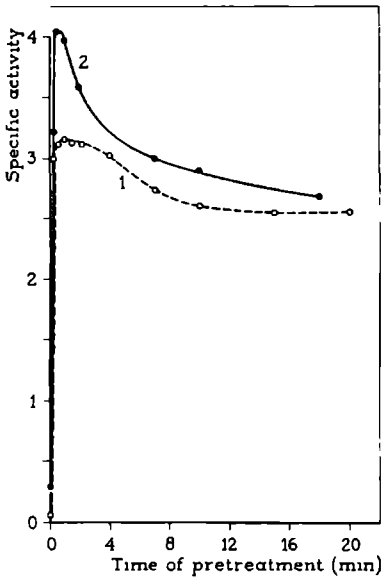


Figure 25

Activation of allantoate amidohydrolase in cell-free extract from *S. allantoicus* (curve 1) or *A. allantoicus* (curve 2) by pretreatment at pH 3.87 and 0°. Cell-free extract (0.8 ml) containing 1.43 (1) or 0.82 (2) mg protein, respectively, in 0.05 M Tris-HCl buffer (pH 7.5), was mixed with 2.4 ml 0.05 M sodium citrate-HCl buffer (pH 3.5). At the indicated time intervals 0.2 ml aliquots of the mixtures were added to 2 ml 0.13 M diethanolamine-HCl buffer (pH 8.8) which contained, per ml, 34.4 µmoles (1) or 23 µmoles (2) sodium allantoate, 0.15 µmole $MnSO_4$ and 5.9 µmoles GSH. Incubation was at 30°. From the amount of allantoate degraded the specific activities of the activated enzyme were calculated.

was adjusted to the same value with HCl or GSH. VOGELS (1966) did not succeed in bringing about an activation of the enzyme at pH values above 4.5; in contrast to the results at pH values below 4.5 this author found an inactivation of previously activated enzyme at pH 6. This inactivation phenomenon will be discussed later (Chapter 7).

6.1.2 *Activation at pH 6*

When the activation experiment given in Fig.25 was repeated at pH 6 and 30° with citrate buffer, a slow but substantial enhancement of the specific activity was obtained, which increased from a value below

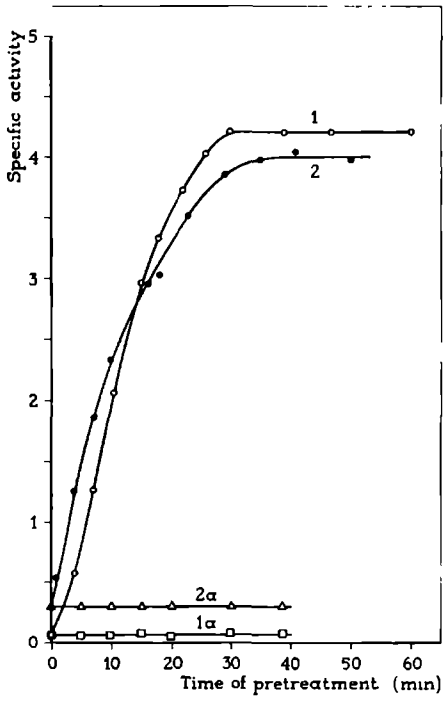


Figure 26

Activation of allantoate amidohydrolase in crude cell-free extract from *S.allantoicus* (curves 1 and 1a) or *A.allantoicus* (curves 2 and 2a) by pretreatment at pH 6.05 and 30°. Cell-free extract (0.8 ml) containing 1.43 (1 and 1a) or 0.82 (2 and 2a) mg protein, respectively, in 0.05 M Tris-HCl buffer (pH 7.5), was mixed with 2.4 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.95). EDTA was added to the preincubation mixtures to a final concentration of 4.25 x 10^{-5}M (curves 1 and 2). Activity was tested as given in Fig. 25.

0.01 to 0.17 unit per mg protein in 30 min. Since this result could be due to a specific effect of this anion, a series of other anions were tested. No enhancement of the specific activity was obtained when one of the following anions was used: phosphate, biphthalate, arsenate, maleate, fumarate, acetate, malonate, succinate, glutarate, adipinate, malate and tartrate (all 7.5 x 10^{-2}M final concentration). However, oxalate dissolved in acetate buffer could replace citrate and moreover yielded a much better result. The specific activity of the enzyme (1 vol.) brought into contact with 0.1 M ammonium oxalate in 0.1 M sodium ace-

tate-acetic acid buffer (3 vol., final pH 6.05) increased from below 0.01 to 1.79 units per mg protein within 10 min at 30°; this rate of activation was more than 10 times higher compared to citrate.

The activating effect of both citrate and oxalate anions at pH 6 could be a result of their property to form cation complexes, although several other anions tested were not devoid of this property. Therefore, it was investigated whether the enzyme was activated when pretreated in a buffer at pH 6 to which a complexing substance, *e.g.* EDTA, was added. The result of such an experiment is shown in Fig.26. No enhancement of the activity was observed in the absence of EDTA. In the presence of 4.25×10^{-5} M EDTA the specific activities of the enzymes from *S.allantoicus* and *A.allantoicus* increased to a maximal value which was of the same order of magnitude as that obtained on pretreatment at pH 3.87 (given in Fig.25). Activation by oxalate and citrate anions was also enhanced by the addition of 4.25×10^{-5}M EDTA: the specific activity had raised from 0.01 to 2.59 in the case of oxalate and from 0.01 to 2.28 in the case of citrate. The activation process at pH 6 was much slower than that at low pH; in the first case maximal activation was obtained after 30 min, whereas in the latter the maximal value was reached within 1 min.

Activation at a pH of about 6 had several distinct advantages over activation at a low pH: 1) milder conditions, thereby diminishing denaturation of the enzyme, 2) after reaching the maximal level no rapid decrease of specific activity occurred and moreover 3) the process of activation was rather slow and thus could be better followed. Therefore, activation procedures were usually performed at pH 6 in this stage of the investigation. The lower activity obtained by pretreatment of the enzyme at pH 3.9 compared to pH 6 was probably due to denaturation of the enzyme; this hypothesis could be confirmed by subjecting purified enzyme material, activated previously at pH 3.9, to activation at pH 6; the latter treatment did not result in a further increase of specific activity. Treatment of purified enzyme material, activated previously at pH 6, for 30 sec at pH 3.9 resulted in a decrease of the specific activity.

In the present study we shall use the term activation mixture to denote that mixture in which the enzyme is converted to the catalytically

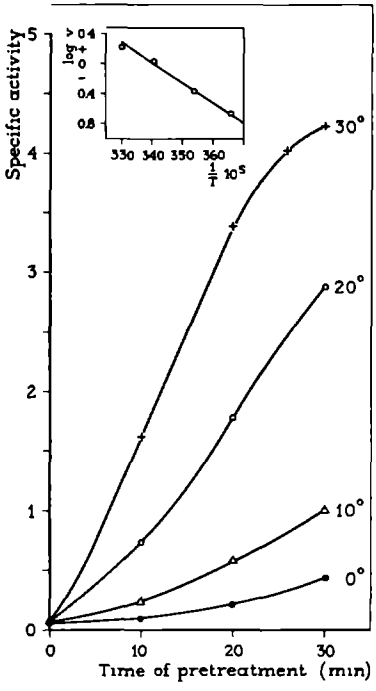


Figure 27

The rate of activation of allantoate amidohydrolase from *S.allantoicus* at pH 6.05 as a function of the temperature. Cell-free extract (0.2 ml) containing 0.71 mg protein in 0.05 M Tris-HCl buffer (pH 7.5, containing 1.7×10^{-4}M EDTA) was added to 0.6 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.95) at different temperatures. At the indicated time intervals the enzymic activity was tested by addition of a 0.2 ml aliquot of the pretreated enzyme to 2.6 ml buffered substrate solution. The final incubation mixtures contained, per ml, 29.6 µmoles sodium allantoate, 4.23 µmoles GSH, 0.14 µmole $MnSO_4$, 120 µmoles diethanolamine-HCl buffer (pH 8.8) and 63.5 µg activated enzyme protein.

active configuration. The term incubation mixture is only applied to the complete activity assay mixture at the optimal pH. Activation is usually performed by mixing 1 vol. of enzyme solution with 3 vol. of the appropriate anion solution and this mixture is treated for a certain time at a certain temperature. At the end of this pretreatment period incubation is started by transferring an aliquot of the activation mixture to 2 ml buffered substrate solution.

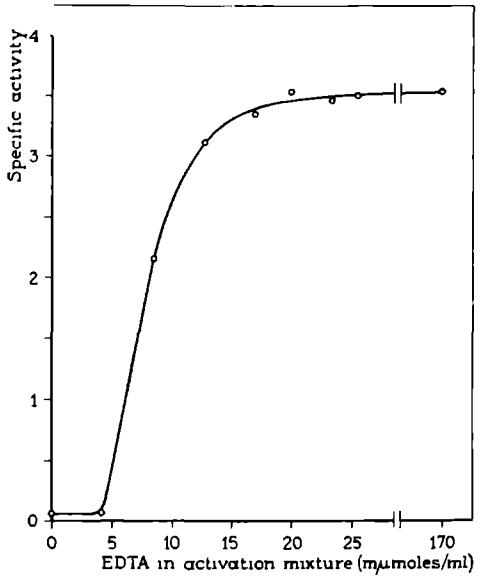


Figure 28

Activation of allantoate amidohydrolase from *S.allantoicus* at pH 6.05 as a function of the EDTA concentration. Cell-free extract (0.2 ml containing 0.34 mg protein and 3.4 mμmoles EDTA) was treated for 30 min at 30° as given in Fig.26. The activation mixtures contained various amounts of EDTA. Incubation was started by addition of 2 ml substrate solution to the activation mixture. The final incubation mixtures contained, per ml, 29.6 μmoles sodium allantoate, 4.23 μmoles GSH, 0.11 μmole $MnSO_4$, 93 μmoles diethanolamine-HCl buffer (pH 8.8) and 0.12 mg of the activated enzyme protein.

6.2 OPTIMAL CONDITIONS FOR ACTIVATION AT pH 6

6.2.1 *Effect of temperature on the activation rate*

Fig. 27 shows the rate of activation of the enzyme at different temperatures during pretreatment in phosphate-EDTA buffer at pH 6.05. At all tested temperatures ranging from 0-30° the activity had increased. From the data in Fig.27 a Q_{10} of 2 and an activation energy of 12 kcal/mole were calculated. In further experiments the enzyme was always activated at 30°. From Fig.26 it already followed that at this temperature maximal activation was obtained after 30 min.

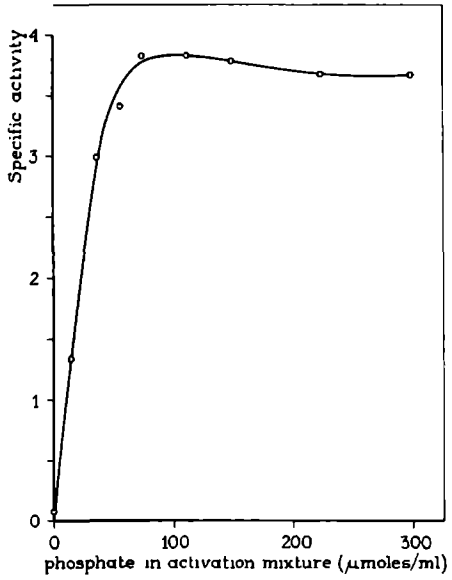


Figure 29

Influence of the molarity of the phosphate buffer during enzyme activation at pH 6.05. Enzyme (0.34 mg protein) was activated with phosphate buffers of different molarities as given in Fig.27. During activation the EDTA concentration was 4.25 x 10^{-5}M. Incubation was started by the addition of substrate solution to the activation mixtures. The composition of the final incubation mixtures is given in Fig.28.

6.2.2 *Effect of the EDTA concentration on the activation*

Activation at a pH of about 6 could be achieved by oxalate or citrate anions; in the presence of EDTA activation also occurred with phosphate anions. Therefore, the influence of the EDTA concentration on the extent of activation after 30 min at pH 6.05 was studied (Fig.28). Below a concentration of 5 x 10^{-6} M EDTA in the activation mixture no increase of the activity was obtained; on increasing the amount of EDTA an enhancement of the specific activity occurred. Maximal activity was obtained at 2 x 10^{-5}M EDTA and further increasing this complexing substance to 1.7 x 10^{-4}M had no effect. This result explained that in the experiments of VOGELS (1966) no activation was observed at pH 6, since no EDTA was present in the phosphate buffers used.

An activation also took place when 1,10-phenanthroline was used instead of EDTA; this complexing agent was about 2.5 times less effective in activation than EDTA.

6.2.3 *Effect of the phosphate molarity during activation*

The influence of the concentration of phosphate in the activation mixture is shown in Fig.29. Even at an optimal EDTA concentration the extent of activation in 30 min was clearly dependent on the molarity of the phosphate buffer (Fig. 29). Optimal activation within 30 min was achieved at phosphate buffer concentrations equal to or larger than 7.5×10^{-2}M. The specific activity was enhanced 63-fold by this treatment. This effect was not due to K^+ and/or Na^+ ions present in the buffer. In a sodium or potassium acetate-acetic acid buffer (final pH 6.05; 4.25×10^{-5}M final EDTA concentration) the specific activity of allantoate amidohydrolase increased too but to a much lesser extent. Even at 1 M sodium acetate-acetic acid buffer the specific activity was enhanced only 9 times within 30 min. In the concentration range 0-1 M sodium acetate-acetic acid buffer (final EDTA concentration 4.25×10^{-5}M) the extent of activation attained within 30 min and also the rate of activation were linearly proportional to the concentration of the buffer.

When oxalate was used as activator anion no difference was found in the rate and ultimate extent of activation in the concentration range 1.5×10^{-2}M - 3×10^{-1}M.

Both phosphate anions and EDTA were necessary for activation at pH 6. Lowering the EDTA concentration to 4.25×10^{-6}M (cf. Fig.28) and increasing at the same time the phosphate molarity up to 0.3 M did not give any activation at all. No further increase of specific activity was obtained when a non-optimal phosphate concentration (1.5×10^{-2}M - 5.6×10^{-2}M) was used and simultaneously the EDTA concentration was enhanced to 10^{-2}M. This EDTA concentration was about 500 times the optimal concentration necessary in the presence of 7.5×10^{-2}M phosphate (cf. Fig.28).

Formerly activation of the enzyme was observed only below pH 4.5. It appeared now that also at pH 6 activation could take place under

proper conditions. From the experiments shown in Figs.26-29 it followed that optimal activation could be achieved by treatment of the enzyme for 30 min at 30° in the presence of 7.5 x 10^{-2}M phosphate and 2 x 10^{-5}M EDTA. The same was true for purified enzyme preparations.

6.3 COMPLEXATION OF CATIONS DURING ACTIVATION AT pH 6

The ability of citrate and oxalate to activate the enzyme at pH 6 could be a result of their complexing power (6.1.2). Other anions also could act as activators when a complexing substance (EDTA) was present during the activation procedure (6.1.2). However, also in the presence of EDTA there was a great difference in the rate and extent of activation of the enzyme, dependent on the anion used and the concentration of this anion; at an EDTA concentration of 4.25 x 10^{-5}M a 63-fold and 2-fold increase of the enzymic activity was obtained with 7.5 x 10^{-2}M phosphate and acetate, respectively. It appeared that EDTA, a strong complexing agent, yielded no appreciable activation of the enzyme: increasing the EDTA concentration from 4.25 x 10^{-5}M to 4.25 x 10^{-4}M did not result in a better activation when acetate (7.5 x 10^{-2}M) was used as anion. The rate of activation, therefore, is determined by the EDTA concentration and to a large extent also by the complexing ability of the other anions of the buffer system. Therefore, we tested a possible relationship between the complexing ability of these anions and the extent and rate of activation of the enzyme at pH 6 under standard conditions (6.2).

6.3.1 *Activation and complexing ability of anions*

6.3.1.1 Activation conditions

In the study on the effect of certain anions on the activation of allantoate amidohydrolase such conditions were used as yielded optimal results in activation with a phosphate-EDTA mixture: 1) pH 6-6.05, 2) a temperature of 30°, 3) an anion molarity during activation of 7.5 x 10^{-2}M, 4) an EDTA concentration of 4.25 x 10^{-5}M and 5) an activation time of 30 min and also 90 min because it could be expected that no

complete activation would be achieved within 30 min in several cases.

6.3.1.2 Measurement of complexation of Mn^{2+} by anions

Complex formation was determined by measuring the percentage of Mn^{2+} ions bound by the anion. Mn^{2+} ions were chosen in this study because this ion displayed an important effect on inactivation (7.1), stabilization (7.3) and furthermore it was the best cofactor for the enzyme (5.5). Purified allantoate amidohydrolase was shown to contain Mn^{2+} by the formaldoxime method (VAN DER DRIFT and VOGELS, 1967). The paramagnetic character of this ion made it possible to measure free Mn^{2+} by using the electron proton resonance technique.

Measurement of the complexation was performed in mixtures containing the anion in the same concentration as used for activation, *viz.* 7.5 x 10^{-2}M, and Mn^{2+} ions to a final concentration of 5 x 10^{-4}M. Although during activation always EDTA (4.25 x 10^{-5}M) was present, this substance was omitted in the complexation studies because we were only interested in the complexing ability of the other anions of the buffer system. In Fig.30 the EPR spectra of Mn^{2+} ions are shown,

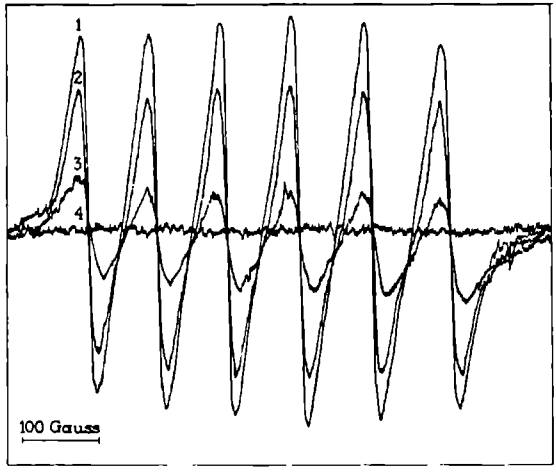


Figure 30

Electron proton resonance spectra of Mn^{2+} ions in the presence and absence of complexing anions at pH 6. Total concentration of Mn^{2+} was 5 x 10^{-4}M; anions were present in a concentration of 7.5 x 10^{-2}M. The spectra represent Mn^{2+} in water (1), sodium acetate-acetic acid buffer (2), KH_2PO_4-Na_2HPO_4 buffer (3) and sodium citrate-citric acid buffer (4).

which were obtained in the presence of certain anions (acetate, phosphate and citrate) (2.2). Since there is a linear relationship between the concentration of free Mn^{2+} ions and the amplitude of the peaks in the EPR spectra, it was possible to calculate directly the percentage of bound Mn^{2+} ions.

Complex formation was so rapid that an equilibrium was reached immediately after addition of Mn^{2+} ions to the anions.

6.3.1.3 Correlation between complex formation and increase of specific activity

Table 9 shows the specific activity of the enzyme observed after treatment with a great number of anions which all were able to bind Mn^{2+} ions. Although it was difficult to establish from the results an absolute correlation between the increase of specific activity and the ability to form complexes, it appeared that generally the greater the complexing power of the anion, the better its ability to activate the enzyme.

6.3.1.4 Initial rate of activation at pH 6

Activation by a phosphate-EDTA mixture yielded maximal activity within 30 min at 30^{o}. From Table 9 it followed that most anions had not brought about maximal activation in this period because the specific activity had attained a higher value after an activation period of 90 min. Therefore, we investigated the rate of activation for a number of anions. In Fig.31 it was shown that the activation rate increased in the order: maleate, phosphate, oxalate. The complex formation with Mn^{2+} ions increased in the same order. For several other anions also the initial rate of activation was measured (Table 9). Again in general a better complexing anion yielded a higher initial rate of activation.

6.4 ACTIVATION OF THE ENZYME AS A FUNCTION OF pH

Since the enzyme allantoate amidohydrolase could be converted from a catalytically inactive to a catalytically active configuration by pretreatment at pH values below 4.5 (VOGELS, 1966; 6.1.1) and by pretreatment under proper conditions at pH 6 (6.1.2), it seemed reasonable to investigate whether activation also took place at other pH values.

Table 9

Effect of various anions on the specific activity of allantoate amidohydrolase and the complexing ability of these anions at pH 6

Activity measurements were performed as described in the text. The composition of the incubation mixture, per ml, was 31.4 μmoles sodium allantoate, 4.3 μmoles GSH, 0.14 μmole $MnSO_4$, 118 μmoles diethanolamine-HCl buffer (pH 8.8) and 0.1 mg activated enzyme protein. The specific activity of the untreated enzyme was 0.28. Complex formation was measured as given in 6.3.1.2.

Anion (7.5×10^{-2}M) 1)	% Mn^{2+} bound by anion 2)	Specific activity (U/mg protein)		Rate of activation (U/mg protein.min)
		30 min	90 min	
Propionate	21	0.53	0.83	
Acetate	28	0.57	0.99	0.0085
Glutamine	36	0.95	1.71	
Glycine	36	1.07	1.32	
Asparagine	38	0.99	1.89	
Fumarate	40	1.29	-	
Adipinate	40	0.55	0.76	0.0043
Glutamate	46	0.96	1.49	
Aspartate	47	1.66	2.10	0.048
Maleate	59	1.39	1.86	0.04
Succinate	60	1.38	1.89	0.033
Biphthalate	73	1.98	1.98	0.06
Phosphate	74	2.04	2.04	0.097
Malate	76	1.45	1.90	0.035
Tartrate	77	2.07	2.12	0.096
Arsenate 3)	80	1.83	1.83	
Malonate	92	2.44	2.44	
Oxalate	92	2.59	2.59	0.57
Glutarate	94	1.00	1.48	0.025
Citrate	100	2.28	2.43	0.33

1) Anions, except biphthalate, phosphate, citrate, glutarate and maleate were dissolved in 0.1 M sodium acetate-acetic acid buffer. Glutarate and maleate were adjusted with 0.1 M Tris to the required pH.

2) Mn^{2+} concentration was 5×10^{-4} M (0 % bound). The spectrum of $MnSO_4$ dissolved in water was similar to that of $MnSO_4$ dissolved in 0.1 M Tris-HCl (pH 7.5).

3) The Mn^{2+}-arsenate mixture used for EPR measurement was slightly turbid. Therefore, the percentage of free Mn^{2+} will be lowered.

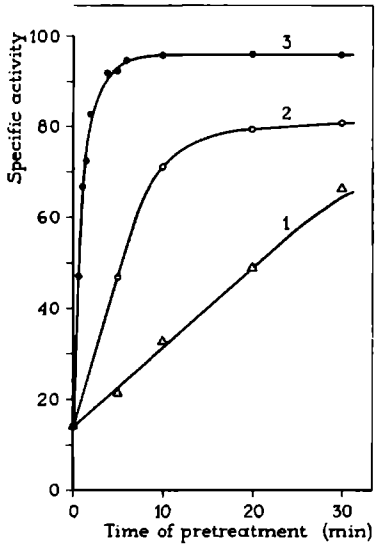


Figure 31

Rate of activation of purified enzyme by treatment at 30° and pH 6.05 with different buffers. Enzyme (1 vol.) was activated by treatment with 3 vol. 0.1 M maleate (1), phosphate (2) or oxalate (3) buffer. During activation the EDTA concentration was 4.25×10^{-5}M. At different time intervals 0.2 ml of the pretreated mixtures was added to 2 ml buffered substrate solution. The incubation mixtures had the same composition as given in Table 9, except they contained 2.3 µg purified enzyme protein.

6.4.1 *Activation at different pH values*

For preliminary experiments such activation conditions were chosen as yielded maximal activation at pH 6 (Fig.32). Maximal activation occurred between pH 6.0 - 6.2. No difference in the extent of activation at all pH values tested was observed when a phosphate, citrate-phosphate or potassium biphthalate-NaOH buffer was used. With crude enzyme preparations a similar curve was obtained at pH values above 6; at lower pH values a much higher activity was obtained which at pH 5.4 still amounted to 90 % of the activity measured at pH 6.05. Therefore, the steep decrease of specific activity at pH values below 6 (Fig. 32) was probably due to denaturation of the purified enzyme. Indeed a much higher specific activity (89) of the purified enzyme was obtained when the activation period at pH 5.25 was shortened to 5 min. The de-

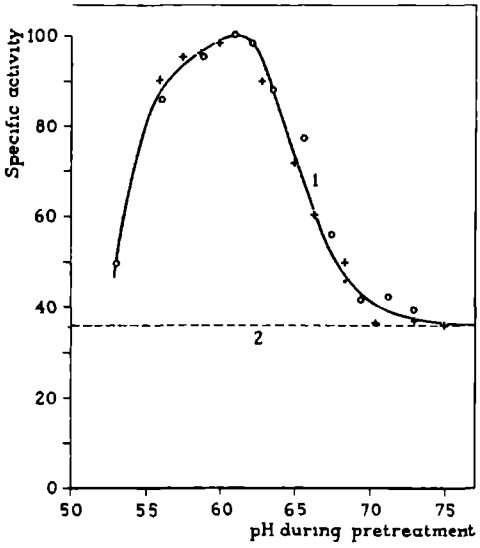


Figure 32

Increase of specific activity of purified allantoate amidohydrolase by pretreatment for 30 min at 30° at various pH values. Purified enzyme (0.2 ml containing 14 µg protein and 34 mµmoles EDTA in 0.05 M Tris-HCl buffer, pH 7.5) was activated by treatment with 0.6 ml 0.1 M Na_2HPO_4 - 0.1 M citric acid buffer (o) or 0.1 M KH_2PO_4 - Na_2HPO_4 buffer (+) with different pH values. Activity tests were performed as given in Fig.28. The specific activity of the untreated enzyme was 36.4.

crease of specific activity at pH values above 6 after treatments for 30 min at 30° could be the result of an incomplete activation or of the establishment of an equilibrium between active and inactive enzyme molecules. Allantoate amidohydrolase, optimally activated at pH 6.05, did not lose any activity on a subsequent treatment for 15 min at pH 6.5, where under normal activation conditions only half of the optimal activity was obtained. This result prompted us to the assumption that the lower values of the specific activity of allantoate amidohydrolase at pH values above 6 were not a result of the establishment of an equilibrium but of an incomplete activation. A further confirmation was obtained by increasing the phosphate molarity during pretreatment at pH 6.5 (Table 10). This resulted in a further enhancement of the specific activity in a similar manner both with a crude and a purified enzyme preparation.

Table 10

Effect of phosphate molarity during activation at pH 6.5

Activation was performed by treatment of 1 vol. enzyme solution (0.43 mg crude or 15 μg purified protein; 34 mμmoles EDTA) with 3 vol. phosphate buffer (pH 6.5) with different molarities for 30 min at 30°. Activity was tested as given in Fig.28. During the activity test 154 μg (crude) and 5.4 μg (purified) activated enzyme protein were present per ml. Maximal specific activity, obtained by activation at pH 6.05, was 3.86 and 100 for crude and purified enzyme, respectively; the specific activity of the untreated enzyme was 0.07 and 36.4, respectively.

Phosphate molarity during activation	Specific activity	
	crude	purified
0.75×10^{-1}	2.03	71.8
1.5×10^{-1}	3.24	84
2.25×10^{-1}	3.34	89
3×10^{-1}	3.62	92

From the experiments described in this paragraph it was evident that the extent of activation obtained within 30 min in phosphate-EDTA buffers depended on the pH of the buffer used. The bell-shape of the curve obtained with purified allantoate amidohydrolase (Fig.32) was determined at the left side by denaturation effects and at the right side by incomplete activation. Therefore, we may conclude that the rate of activation depends on the pH of the buffer used. To obtain optimal activation at higher pH values one must incubate for a longer period.

6.4.2 *Plateau of activation as a function of pH*

From Table 9 it followed that several anions (phosphate, biphthalate, arsenate, malonate and oxalate) under the experimental conditions had brought about maximal activation of the enzyme within 30 min. Oxalate yielded the highest plateau of activity (see also Fig.31). In phosphate buffer a 15-20 % lower value was obtained. This was possibly due to an inhibition by phosphate anions either during activation of the enzyme or during the activity test. The latter possibility was tested by addition of phosphate to the activity test medium of enzyme material activated by oxalate-EDTA. The final concentration of phosphate was equal to that

present in incubation mixtures of enzyme activated by phosphate-EDTA buffer. No inhibition of phosphate on the activity of allantoate amidohydrolase was observed.

To study the maximal extent (plateau) of activation at different pH values oxalate anions were used as complexing anions.

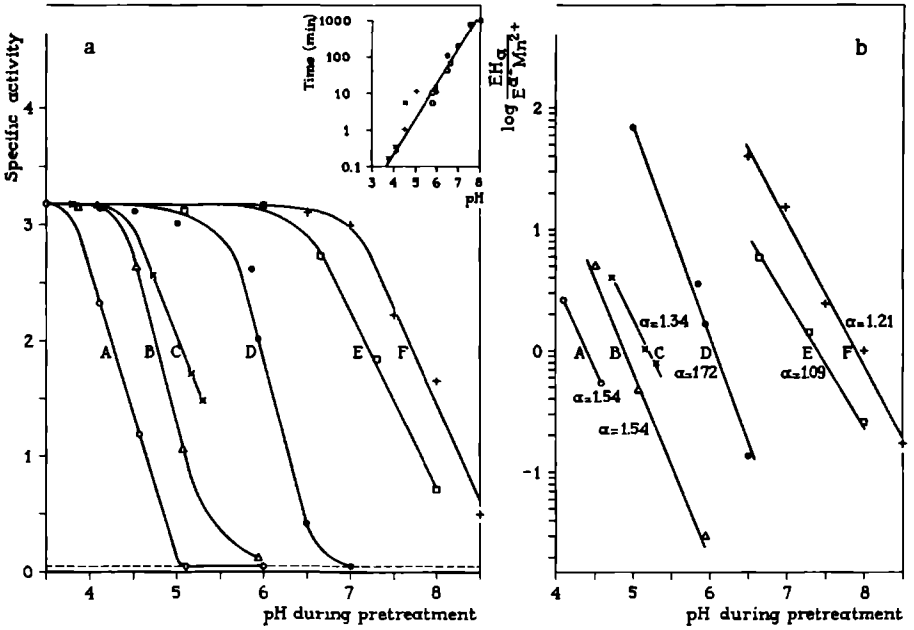


Figure 33a

Plateau of activation as a function of pH. 0.4 ml crude enzyme solution (1.4 mg protein in 0.05 M Tris-HCl buffer, pH 7.5) was treated at 30° with 1.2 ml of the appropriate buffer system. For curve A: 0.1 M sodium acetate-acetic acid; B: 2 x 10^{-3}M oxalate in 0.1 M acetate buffer; C: 5.65 x 10^{-5}M EDTA in 0.1 M acetate buffer; D: 0.1 M oxalate in 0.1 M acetate buffer; E: 0.1 M oxalate + 5.65 x 10^{-5}M EDTA in 0.1 M acetate or 0.1 M Tris-HCl buffer and F: 0.1 M oxalate + 6.75 x 10^{-4}M EDTA in 0.1 M acetate or Tris-HCl buffer. Activity was tested by the addition of 0.2 ml of the pretreated mixture to 2 ml substrate solution containing, per ml, 31.4 μmoles sodium allantoate, 4.72 μmoles GSH, 0.15 μmole $MnSO_4$ and 130 μmoles diethanolamine-HCl buffer (pH 8.8). The inset of the figure represents the time of pretreatment necessary to achieve the establishment of the equilibrium at the different pH values.

Figure 33b

Determination of the number of protons involved in the activation process.

6.4.2.1 Establishment of the plateau of activation under different conditions

Activation is considered a removal of Mn^{2+} ions from the catalytically inactive enzyme. VOGELS (1966) observed that an equilibrium was reached between active and inactive molecules of the enzyme from *E.coli* var. *acidilactici*, when treated at pH values below 4.5. The equilibrium position was pH-dependent. With the enzyme from *S.allantoicus* a similar behavior was observed, although the establishment of an equilibrium was not so evident from his results.

In Fig.33a the maximal extent of activation of allantoate amidohydrolase is given as a function of pH under different conditions. To get an impression of the time necessary to reach this plateau in the inset of Fig.33a this time is plotted as a function of pH. The establishment of the equilibrium was dependent on the pH during activation and the complexing ability of the activation mixture. In the presence of acetate anions an activation occurred only below pH 5 (curve A), but addition of a complexing substance, *e.g.* EDTA (curve C) or oxalate (curves B and D) shifted the equilibrium between active and inactive enzyme in the direction of the active enzyme at a certain pH. This effect was still more evident when both oxalate and EDTA were added (curves E and F). The displacement of Mn^{2+} ions by H^+ ions can be considered the primary reaction during activation; the second reaction will consist in binding of the free Mn^{2+} ions by complexing substances present in the activation mixture. This reaction will be dependent on the concentration of complexing substances; both oxalate (curves B and D) and/or EDTA (curves C, E and F) influence the equilibrium position.

If the primary reaction indeed consisted in a removal of Mn^{2+} ions by binding of protons then the following equations must be true:

$$\underset{\text{inactive enzyme}}{E^{a}\text{-}Mn^{2+}} + aH^+ \rightleftharpoons \underset{\text{activated enzyme}}{EH_a} + Mn^{2+} \qquad (1)$$

$$K = \frac{[EH_a]\,[Mn^{2+}]}{[E^{a}\text{-}Mn^{2+}]\,[H^+]^a} \qquad (2)$$

$$\frac{[EH_a]}{[E^{a}\text{-}Mn^{2+}]} = \frac{K}{[Mn^{2+}]}\,[H^+]^a \qquad (3)$$

$$\log \frac{[EH_a]}{[E^a\text{-}Mn^{2+}]} = \log \frac{K}{[Mn^{2+}]} + a \log [H^+] \quad (4)$$

$$\log \frac{[EH_a]}{[E^a\text{-}Mn^{2+}]} = \log \frac{K}{[Mn^{2+}]} - a \times pH \quad (5)$$

In Fig.33b $\log \frac{[EH_a]}{[E^a\text{-}Mn^{2+}]} = \log \frac{[\text{activated enzyme}]}{[\text{inactive enzyme}]}$ is plotted as a function of the pH during activation. From the slope of the curves obtained the number of protons (a) involved in the activation reaction could be calculated. The factor $\frac{K}{[Mn^{2+}]}$ appeared to be a constant for a certain complexing system. The mean value of a, which represented the number of protons involved in displacement of Mn^{2+} ions, was 1.54 in the pH region 4-6. This value was used in the following calculations.

If $\frac{[EH_a]}{[E^a\text{-}Mn^{2+}]} = 1$, *viz.* if half of the enzyme molecules was in the catalytically active configuration, then it would follow from equation (5) that:

$$\log \frac{K}{[Mn^{2+}]} = a \times pH \quad (6)$$

Calculation for curve A (Fig. 33a) would yield:

$$\log \frac{K}{[Mn^{2+}]_A} = 1.54 \times 4.4 = 6.78 \quad (7)$$

$$\frac{K}{[Mn^{2+}]_A} = 6.03 \times 10^6 \quad (8)$$

The same calculation applied to curve D (Fig. 33a) would yield:

$$\log \frac{K}{[Mn^{2+}]_D} = 1.54 \times 6.05 = 9.31 \quad (9)$$

$$\frac{K}{[Mn^{2+}]_D} = 2.04 \times 10^9 \qquad (10)$$

In the latter case oxalate was present as complexing anion:

$$Ox^{2-} + Mn^{2+} \rightleftharpoons MnOx \qquad (11)$$

$$K_{complex} = \frac{[MnOx]_D}{[Ox^{2-}]_D [Mn^{2+}]_D} \qquad (12)$$

The value of the association constant of the manganous - oxalate complex was 7.8×10^3 (GURD and WILCOX, 1956). From equations (8) and (10) it followed that:

$$K = 6.03 \times 10^6 \times [Mn^{2+}]_A \qquad (13)$$

$$\text{and } K = 2.04 \times 10^9 \times [Mn^{2+}]_D \qquad (14)$$

The assumption was made that $[MnOx]_D \ll [Ox^{2-}]_D = 7.5 \times 10^{-2}$ and that $[Mn^{2+}]_D \ll [MnOx]_D = [Mn^{2+}]_A$, since in both cases half of the maximal activation was achieved; consequently, the total Mn^{2+} split off from the enzyme must be the same in both cases. Substitution in equation (14) yielded:

$$K = 2.04 \times 10^9 \times \frac{[Mn^{2+}]_A}{7.5 \times 10^{-2} \times 7.8 \times 10^3} = 3.5 \times 10^6 \times [Mn^{2+}]_A \qquad (15)$$

The discrepancy between the K values given in equations (13) and (15) was not surprising because the value of the protons, a, involved in the activation reaction could not be determined exactly, and from equation (6) it was clear that a small shift in this value would have a relatively great influence on the calculated value of K. Therefore, it appeared that K defined as given in equation (1) was rather independent on the pH during activation. If we were able to determine the concentration of free Mn^{2+} ions in the activation mixture, it would be possible to calculate the equilibrium constant of equation (2) by using equation (6). However, we were not able to determine this concentration at the present time, since it was too small.

In conclusion we can say that both pH and complexing agents influ-

enced the equilibrium between active and inactive enzyme molecules. By measuring the equilibrium positions it was possible to calculate approximately the number of protons, which were bound to the enzyme during removal of Mn^{2+} ions. It was evident that the time necessary to reach the equilibrium position was longer at higher pH values and furthermore depended on the complexing ability of the activation mixture. Therefore, the initial rate of activation under different conditions was studied as a function of pH.

6.4.3 *Rate of activation as a function of pH*

The velocity of activation of the enzyme at different pH values and under different conditions is shown in Fig.34a. It was evident that both pH and complexing ability during activation determined the initial activation rate. Using equation (1) (6.4.2.1):

$$E^{a-}Mn^{2+} + aH^{+} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EH_a + Mn^{2+} \qquad (1)$$

inactive enzyme activated enzyme

it followed that the velocity of activation was given by:

$$v = k_1 \left[E^{a-}Mn^{2+}\right]\left[H^{+}\right]^{a} \qquad (16)$$

Since k_1 and also $\left[E^{a-}Mn^{2+}\right]$ in the initial phase of activation are fixed values, equation (16) could be read as:

$$v = b\left[H^{+}\right]^{a}, \text{ where } b = k_1\left[E^{a-}Mn^{2+}\right] \qquad (17)$$

$$\log v = a \log \left[H^{+}\right] + \log b \qquad (18)$$

$$\log v = - a \times pH + \log b \qquad (19)$$

In Fig.34b log v is plotted as a function of pH. From the slope of the curves obtained the number of protons, a, involved during activation could be calculated. A reasonable agreement existed between these values and those calculated by equilibrium determinations as given

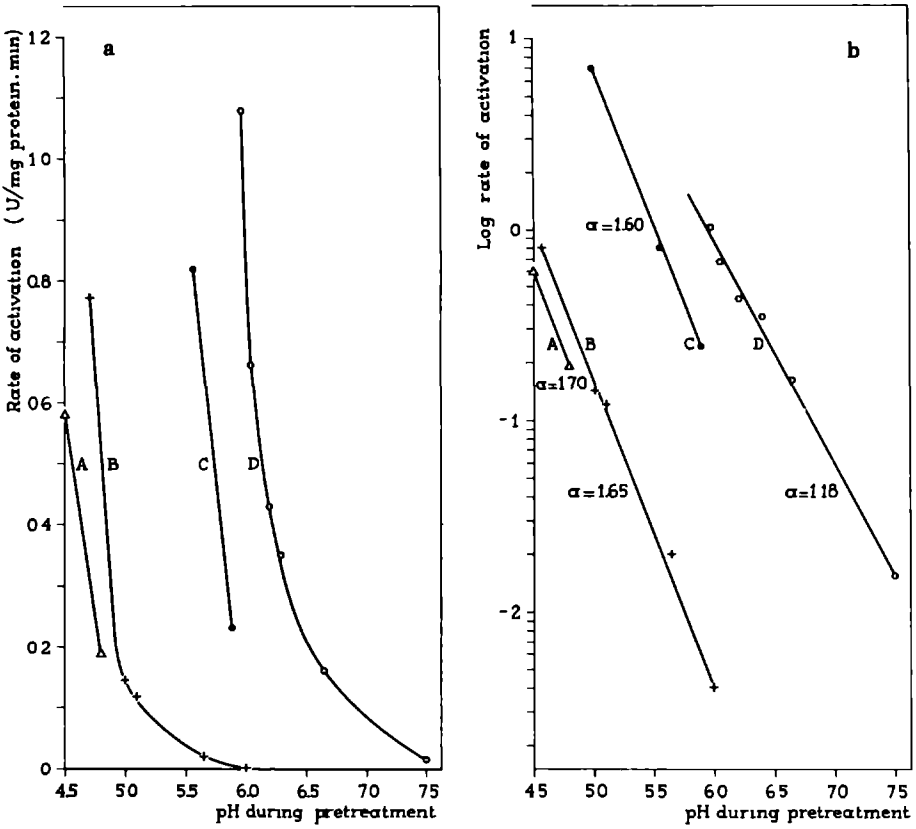


Figure 34a

Rate of activation as a function of pH. 0.4 ml crude enzyme preparation (1.4 mg protein in 0.05 M Tris-HCl buffer, pH 7.5) was treated at 30° with 1.2 ml of the appropriate buffer system. For curve A: 0.1 M sodium acetate-acetic acid buffer; B: 5.65 x 10^{-5}M EDTA in 0.1 M acetate buffer; C: 2 x 10^{-3}M oxalate + 5.65 x 10^{-5}M EDTA in 0.1 M acetate buffer and D: 0.1 M oxalate + 5.65 x 10^{-5}M EDTA in 0.1 M acetate or 0.1 M Tris-HCl buffer. Activity was tested as given in Fig. 33a.

Figure 34b

Determination of the number of protons involved in the activation process.

in Fig.33b. The factor b of equation (17) was a measure for the complexing ability of the activation mixture. Both from Figs. 33b and 34b it followed that fewer protons were involved in the activation reaction at pH values above 6 compared to lower pH values. This might be explained by assuming a change of the charge of one of the groups in the

active center of the enzyme, which are involved in binding of Mn^{2+} ions.

In Fig.35 the effect of EDTA on the rate of activation at pH 7.5 in the presence of 7.5 x 10^{-2}M oxalate is represented. At low EDTA concentrations (below 2 x 10^{-4}M) the initial rate of activation was dependent on this concentration, but above 2 x 10^{-4}M EDTA the rate was constant. In this range the velocity of activation will depend on the H^+ ion concentration and possibly on the molarity of the oxalate anions.

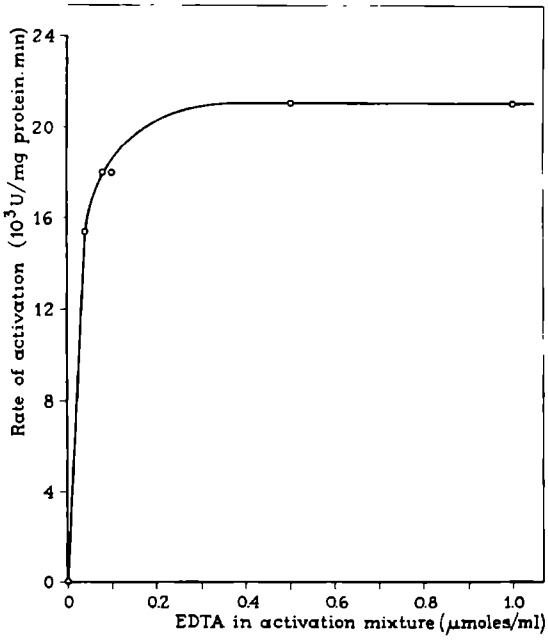


Figure 35

Effect of EDTA on the activation rate at pH 7.5 in the presence of oxalate. 0.3 ml crude enzyme solution (1 mg protein in 0.05 M Tris-HCl buffer) was treated with 0.9 ml 0.1 M Tris-HCl-oxalate buffer (pH 7.5). Final concentration of oxalate was 7.5 x 10^{-2}M. EDTA was present in the activation mixture in the concentrations indicated in the figure. Activity was tested as given in Fig.33a.

6.5 EFFECT OF ALLANTOATE AND GLUTATHIONE ON THE ACTIVATION AT pH 6.05

In Fig. 36 the effect of the substrate and the cofactor GSH on the process of activation is shown. It appeared that upon addition of GSH the activation process almost immediately stopped. No explanation of this phenomenon can be given at present. Allantoate lowered the activation rate and presumably also the activation plateau. Since allantoate will bind to the active center, it is likely to assume that the Mn^{2+} ions, which have to be removed from the inactive enzyme, are shielded in the presence of the substrate. Therefore, the incorrectly bound cations must be bound at or very near to the active center. Perhaps the same explanation can be given for the phenomenon observed with GSH.

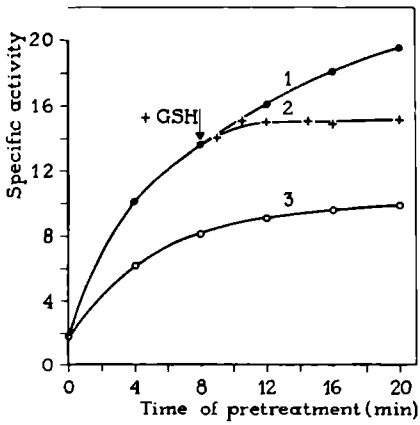


Figure 36

Effect of allantoate and GSH on the activation at pH 6.05. 0.5 ml enzyme solution (0.17 mg protein in 0.05 M Tris-HCl buffer, pH 7.5) was treated with 3 vol. 0.1 M KH_2PO_4-Na_2HPO_4 buffer. EDTA was present in a concentration of 4.2×10^{-5}M during activation. To obtain curve 2 at t = 8 min GSH was added to a final concentration of 4.2×10^{-3}M. For curve 3 allantoate was added at the start of the activation period to a final concentration of 1.34×10^{-2}M. Activity was tested as described in Fig.33a.

6.6 ACTIVATION BY SALT SOLUTIONS

VOGELS (1966) observed that activation occurred also by pretreatment of the enzyme with certain salt solutions at pH 4.9. The increase of activity was dependent on the molarity of the salt solution. We studied this effect at pH 6 with Na^+ or K^+ salts of the halogens. The fluorides were not included, since it appeared that F^- was an inhibitor of the enzymic activity (5.8). After an activation period of 30 min at 30° NaBr and NaI (7.5×10^{-2}M) yielded the highest extent of activation; all salts were dissolved in 0.1M acetate buffer and the pH was adjusted to 6.
The influence of the NaBr molarity on the activation at pH 6 was studied (Table 11). A higher extent of activation was achieved with higher NaBr molarities; higher concentrations of NaBr than used could not be tested since a limit was set by the solubility of NaBr. VOGELS

Table 11

Activation of allantoate amidohydrolase from *S. allantoicus* by pretreatment in solutions containing different molarities of NaBr at pH 6

0.2 ml crude enzyme solution (0.56 mg protein and 34 mμmoles EDTA in 0.05 M Tris-HCl buffer, pH 7.5) was treated for 30 min at 30° with 0.6 ml 0.1 M acetate buffer (pH 5.8) containing the salt; the pH values of the salt solutions were checked and adjusted to pH 5.8, if necessary. Final pH during activation was 6. Incubation was started by the addition of 2 ml buffered substrate solution. The incubation mixtures contained, per ml, 29.6 μmoles sodium allantoate, 4.23 μmoles GSH, 0.14 μmole $MnSO_4$, 120 μmoles diethanolamine-HCl buffer (pH 8.8), 0.2 mg activated enzyme protein and NaBr in the range between 0-1.44 M.

NaBr molarity during pretreatment	Increase of specific activity (U/mg protein.30 min)
-	0.04
0.315	0.5
0.63	0.66
1.26	0.785
2.52	1.15
3.77	1.46
5	2

(1966) observed an optimal activation at 3 M NaBr and the extent of activation decreased at higher concentrations. A possible explanation may be the difference in pH during activation since this author used pH 4.9 and an activation period of 2 min at room temperature. The enzyme may be more susceptible to denaturation by high salt molarities at a lower pH.

The activation by NaBr, observed after pretreatment at pH 6, could be due to an enhancement of the activity of the enzyme in the subsequent activity test. Therefore, the effect of NaBr on the enzymic activity was studied also at the optimal pH (Fig.37). Activation occurred also at this pH. Pretreatment of the enzyme in 5 M NaBr for 10 min and 30 min enhanced the specific activity from 0.06 to 0.77 and 0.83, respectively.

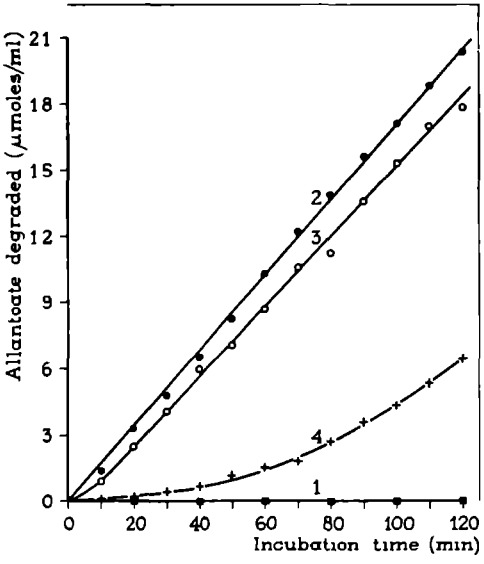


Figure 37

Effect of sodium bromide on the activity of allantoate amidohydrolase from *S. allantoicus* at pH 8.8. 0.2 ml crude enzyme solution (0.56 mg protein and 34 mμmoles EDTA in 0.05 M Tris-HCl buffer, pH 7.5) was treated for 30 min (curves 1 and 2) or 10 min (curve 3) at 30° with 0.6 ml 0.1 M diethanolamine-HCl buffer (pH 8.8) containing NaBr. During this pretreatment the NaBr was 5 M. Activity was tested by the addition of 2 ml substrate solution. The incubation mixture had the composition, per ml, as given in Table 11. NaBr in the assay mixtures was 5 M (curve 1) and 1.44 M (curves 2, 3 and 4). The enzyme solution tested in curve 4 was not pretreated.

In the activity test medium NaBr was diluted to 1.44 M. No enzymic activity was measured when the enzyme was tested in 5 M NaBr. Direct incubation in the presence of 1.44 M NaBr resulted also in an enhancement of the activity.

It was clear that activation by salt solutions depended on the time of pretreatment and the pH and molarity of the salt solution. No effect of EDTA (4.25×10^{-5}M) on the extent of activation by salt solutions was observed.

It is known that the concentration of free Mn^{2+} ions decreases with increasing chloride concentrations (O'SULLIVAN and COHN, 1966). Activation by high molarities of salts could be due to complexation of Mn^{2+} or more likely to a change in the charge distribution of the enzyme.

6.7 DISCUSSION AND CONCLUSIONS

Survey of activation phenomena

The phenomenon that an enzyme can be present in an active and an inactive configuration is well-known in enzymology. For example a number of proteolytic enzymes is known both in the inactive and active form (DIXON and WEBB, 1964): carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.2.1), pepsin (EC 3.4.4.1), rennin (EC 3.4.4.3), trypsin (EC 3.4.4.4), chymotrypsin (EC 3.4.4.5) and thrombin (EC 3.4.4.13). The inactive configuration or precursor of the enzyme is converted into the active enzyme by catalytic action of either hydrogen ions or enzymes. This activation appears to consist in a breaking of peptide links, with or without removal of free peptides.

Some enzymes are inactive since they occur as an enzyme-inhibitor complex, *e.g.* NAD pyrophosphatase (dinucleotide nucleotidohydrolase, EC 3.6.1.9) in extracts of *Proteus vulgaris*. The inhibitor was heat- and acid-labile, whereas the enzyme was stable in both treatments (SWARTZ, KAPLAN and FRECH, 1956). NADase (NAD glycohydrolase, EC 3.2.2.5) from *Mycobacterium tuberculosis* was inactive in cell-free extracts because of the presence of an inhibitor (GOPINATHAN, SIRSI and VAIDYANATHAN, 1964).

The activation phenomenon of allantoate amidohydrolase was clearly distinguished from those observed with proteolytic enzymes. In the first place activation of allantoate amidohydrolase by H^+ ions was very rapid at low pH, usually being complete within 30 sec at 0°. Secondly, the pH region in which the acid-activation occurred (below pH 5) was far from the pH optimum (pH 8.5-9.5) of the enzyme. Thirdly, no evidence was obtained for the removal of low- or high-molecular parts. This excluded also the presence of an enzyme-inhibitor complex. In the fourth place activation could be achieved by using complexing substances. In the next Chapter we will see that inactivation of the enzyme was achieved by cations and that the processes of activation and inactivation were reversible. All these facts taken together led us to the conclusion that activation of allantoate amidohydrolase was based on a mechanism different from that responsible for the activation of proteolytic enzymes or enzyme-inhibitor complexes.

Some other, non-proteolytic, enzymes could be activated at low or high pH values.

Dihydropyrimidinase (4,5-dihydropyrimidine amidohydrolase, EC 3.5.2.2) from calf liver was reported (WALLACH and GRISOLIA, 1956) to be activated by treatment of crude liver extract with 0.1 N acetic acid.

Latent phenolase (*o*-quinol: oxygen oxidoreductase, EC 1.10.3.1) from different sources (Drosophila species, *Tenebrio molitor*, *Vicia faba* L.) could be activated by H^+ or OH^- ions (KENTEN, 1957). The specific activity of the enzyme was also enhanced by treatment with various denaturing agents (HEYNEMAN and VERCAUTEREN, 1964).

3-Hydroxyanthranilate oxygenase (3-hydroxyanthranilate: oxygen oxidoreductase, EC 1.13.1.6) could be activated by treatment at pH 4 in the presence of Fe^{2+} ions and cysteine (OGASAWARA, GANDER and HENDERSON, 1966).

Homogentisate oxygenase (homogentisate: oxygen oxidoreductase, EC 1.13.1.5) from rat liver had to be treated at pH 4 in the presence of Fe^{2+} ions to reach maximal enzymic activity (TOKUYAMA, 1959). The enzyme from *P. fluorescens* needed for maximal activation a pretreatment at pH 6.0 in the presence of Fe^{2+} (ADACHI, IWAYAMA, TANIOKA and TAKEDA, 1966).

Table 12

Activation of non-proteolytic enzymes by pH treatment

Enzyme	Mode of activation	pH optimum	Suggested mechanism	Reference
NAD pyrophosphatase	10 min at pH 2 and room temperature	7.5	Removal of an inhibitor	SWARTZ, KAPLAN and FRECH, 1956
Dihydro-pyrimidinase	0.1 N acetic acid	8.5 (substrate: hydrouracil) 5.5 (substrate: carbamoyl-β-alanine)	Removal of inhibitor or enzyme fragmentation	WALLACH and GRISOLIA, 1956
Phenolase	Brief exposure to pH 3.5 or pH 11.5 at 5-10°	6	Removal of inhibitor or activation of a precursor or a conformational change	KENTEN, 1957 HEYNEMAN and VERCAUTEREN, 1964
3-Hydroxy-anthranilate oxygenase	5 min at pH 4 and room temperature in the presence of Fe^{2+} and cysteine	6.5	Binding of Fe^{2+} to the apoenzyme	OGASAWARA *et al.*, 1966
Homogentisate oxygenase	5 min at pH 4 and 0° or 10 min at pH 6 and 30°, both in the presence of Fe^{2+}	6	Binding of Fe^{2+} to the apoenzyme	TOKUYAMA, 1959 ADACHI *et al.*, 1966
Glucose-6-phosphate phosphatase	10 min at pH 9.5-9.8 and 0°	6.1	Unknown	STETTEN and BURNETT, 1966
Allantoinase	6 min at pH 4.5-5.4 and 0°	7.5-8.5	Unknown	VOGELS *et al.*, 1966

The activities of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) and the related activities of inorganic pyrophosphatase and inorganic PP_i-glucose phosphotransferase were maximally increased by treatment at pH 9.5-9.8. Activation was not a result of solubilization of the enzyme (STETTEN and BURNETT, 1966).

Activation of mitochondrial cytochrome oxidase (cytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) was due to solubilization of the enzyme at alkaline pH values (PERSON *et al.*, 1965). The apparent increase of enzymic activity as a result of solubilization is known for more enzymes, *e.g.* latent acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) from chloroplasts (RAGETLI, WEINTRAUB and RINK, 1966) and erythrocyte membrane ATPase (ATP phosphohydrolase, EC 3.6.1.3; CHAN, 1967), but we shall not discuss this effect here since it is evident that activation of allantoate amidohydrolase bears no resemblance at all to this phenomenon.

Allantoinase (allantoin amidohydrolase, EC 3.5.2.5) from higher plants was slightly activated by treatment at pH 4.5-5.4 (VOGELS, TRIJBELS and UFFINK, 1966).

A summary of the above-described activation effects on non-proteolytic enzymes is represented in Table 12. It is remarkable that all enzymes summarized were activated by bivalent cations or even contained them as an integral part of the enzyme molecule. The same was true for allantoate amidohydrolase. This enzyme had an absolute requirement for Mn^{2+} ions at the pH optimum (5.5).

The activation phenomenon with allantoate amidohydrolase

It was known that the activity of allantoate amidohydrolase, which was low in cell-free extracts, could be enhanced by a short pretreatment at pH values below 4.5. Furthermore, there appeared to exist an equilibrium between active and inactive enzyme molecules at these pH values: this was evident especially for the enzyme from *E. coli* var. *acidilactici* (VOGELS, 1966).

In our study we demonstrated that the enzyme could also be activated at pH values above 4.5; even at pH 8.5 an activation of the enzyme could be achieved. However, if the pH during the activation treatment was increased from pH 4.5 to higher pH values the complexing ability of the

activation mixture must also increase to obtain activation. Oxalate and to a slight extent also citrate anions were able to act as activators of the enzyme at pH values above 4 up to pH 8.5. Other complexing anions were able to perform activation at pH values above 4, but only in the presence of an extra complexing agent, *viz.* EDTA. This substance still enhanced the extent of activation obtained by using oxalate or citrate anions as activators.

Activation by complexing anions and activation by treatment at low pH led us to the assumption that cations were split off from the enzyme during activation. A special function was ascribed to Mn^{2+} ions, since the presence of these ions in purified enzyme preparations was demonstrated. Mn^{2+} ions were the best cofactors of the enzyme. Furthermore these ions could inactivate active or activated enzyme and were able to stabilize the enzyme above pH 8 (cf. Chapter 7). In conclusion we can say that most likely Mn^{2+} ions were involved in the activation process. Since Mn^{2+} ions were essential for catalytic action of the enzyme, it appeared that the Mn^{2+} ions removed during activation were bound in such a position in the inactive enzyme that the active center of the enzyme was not able to bring about catalysis. Substrate inhibited the rate of activation and therefore it was likely that the cation itself was bound in the active center of the enzyme molecule.

In the light of this hypothesis activation consisted in removal of incorrectly bound Mn^{2+} ions from particular ligands of the enzyme. Activation could be performed by treatment at low pH or by means of a complexing buffer system. The latter activation was dependent on the complexing ability of the anions (buffer anions and/or EDTA) of the buffering system and of the pH during activation. Below pH 4 addition of EDTA did not result in a better activation (both the rate and plateau of activation were determined). At these pH values EDTA will not be able to complex Mn^{2+} ions and consequently will have no effect on the equilibrium between active and inactive enzyme molecules. At pH values above 4 EDTA exerted an effect on the position of this equilibrium and also on the rate of activation. The equilibrium was shifted in the direction of the active enzyme molecules and the rate of activation was enhanced. At pH 7.5 the enhancing effect of EDTA on the rate of activation was only observed at concentrations up to 2×10^{-4}M.

By using the EPR technique we were able to correlate the extent of complexation of Mn^{2+} ions by the anion with the extent of activation of the enzyme obtained in the presence of the same anion at pH 6. In general in a certain period of time better activation was obtained with a better complexing anion. Measurements of the rate of activation revealed that also a higher velocity of activation was obtained at pH 6 when a better complexing anion was used. Of course there is a large difference between binding of Mn^{2+} ions in a model system only containing the cations and the buffer anions at the desired pH and binding of the Mn^{2+} ions in a system also containing besides the buffer anions EDTA and enzyme molecules. In the latter case the factor steric hindrance may be involved, because some buffer anions which can complex Mn^{2+} reasonably well in model systems, are perhaps not able to approach closely enough to remove Mn^{2+} bound to the enzyme. This seemed to get evidence from the observation that some anions, which were able to complex Mn^{2+} ions well in model systems, were not able to bring about an as high activation as could be expected from their complexing ability.

Mn^{2+} ions were assumed to be bound in the active center of the enzyme. Treatment at a pH below 5 removed these ions from the enzyme and presumably a carboxyl group was involved in the binding of Mn^{2+}. This would implicate that a carboxyl group was present in the active center.

The extent and rate of activation was dependent on the pH during activation and furthermore on the concentration of complexing substances. The equilibrium constant K appeared to be rather independent on the pH during activation.

Activation could be represented as:

$$E^a\text{-}Mn^{2+} + aH^+ \rightleftharpoons EHa + Mn^{2+} \qquad (1)$$

inactive enzyme activated enzyme

$$Mn^{2+} + C \rightleftharpoons MnC, \text{ where } C = \text{complexing anion} \qquad (20)$$

$$MnC + EDTA \rightleftharpoons MnEDTA + C \qquad (21)$$

In the activity test medium at pH 8.8:

$$\underset{\text{activated enzyme}}{EH_a} + Mn^{2+} \rightleftharpoons \underset{\text{active enzyme}}{{}^*E^a\text{-}Mn^{2+}} + aH^+ \qquad (22)$$

During activation H^+ ions displaced Mn^{2+} ions from the inactive enzyme resulting in an activated enzyme, which exerted catalytic activity when tested at the pH optimum in the presence of Mn^{2+}. The primary reaction is assumed to be the displacement of Mn^{2+} from the enzyme ligands by H^+ ions. The released Mn^{2+} is presumably first complexed to the anion, since oxalate and citrate are able to bring about activation of the enzyme in the absence of EDTA. The function of EDTA then only will consist in a trapping of those ions which were released from the Mn^{2+}-anion complex and otherwise could again bind to the activated enzyme, which would result in an inactivation. In the case of anions other than oxalate and citrate EDTA is necessary to achieve any activation. To achieve maximal activation the concentration of the anions must be above certain levels. At least 2×10^{-5}M EDTA was required for optimal activation when phosphate anions were used as activator anions; moreover, the phosphate anion concentration had to be at least 7.5×10^{-2}M. When one of the concentrations of EDTA or phosphate was lower than the given value and the other one was far above this value no optimal activation was obtained. Therefore, the requirements for the concentrations of both substances are independent of each other. A possible explanation of these results can be given by ascribing a transport function to the anion which transfers the cation to the outside of the enzyme. EDTA complexes the cation but is itself not able to remove wrongly bound cations from the enzyme.

Activation by high molarities of salts could not be due only to a complexation of Mn^{2+} ions, since there was a difference (about 20%) in the extent of activation by 1 M NaBr and 1 M KBr at pH 6. The activation effect may be explained by assuming a change in the charge distribution on the enzyme, thereby leading to some conformational change which favored the splitting off of Mn^{2+} ions from the enzyme. Complexing agents like EDTA had no influence on the extent of activation by salt solutions.

Activation by EDTA

Preincubation with EDTA enhanced also the activity of phosphoglucomutase (α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1). Mg^{2+} ions exerted an effect similar to that of EDTA. This activation consisted in the removal of inhibitory metal ions by chelation or replacement of the inhibiting ion by Mg^{2+} in the presence of histidine or imidazole (RAY and ROSCELLI, 1966). The preincubation was performed at the same pH as was used in the assay mixtures for enzymic activity in contrast to our experiments with EDTA.

Activation by anions

Activation by anions is an effect which is encountered with several enzymes and is mostly unspecific. As early as in 1904 it was demonstrated (COLE, 1904) that the activity of some enzymes was influenced by anions; the most striking effect was observed with chloride ions on the activity of α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) (MYRBÄCK, 1926). Influence by bi- and trivalent anions was met with fumarase (L-malate hydro-lyase, EC 4.2.1.2) (MANN and WOOLF, 1930). These anion effects were due to an effect on the ionization of some groups at the active center of the enzyme, which determined the optimal pH (MASSEY, 1953). Anions may affect the enzymic activity also by another mechanism. The combination of enzyme and substrate may be altered. Several years ago it has been found (THEORELL, 1958) that the affinity of NADH for alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1) in the presence of chloride ions had decreased.

For arylsulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) which was also activated by anions it was demonstrated (WEBB and MORROW, 1959) that no shift in the pH-activity curve and no effect on the affinity to the substrate occurred. An increase of the velocity of the reaction was observed, *viz.* an increased rate of breakdown of the enzyme-substrate complex.

The possibility that all these effects could be attributed to the binding of anions to the enzyme either at the catalytically active or at another site was suggested by ALBERTY (1956). Indeed such an effect was ob-

served for several enzymes, *e.g.* phosphate binding to fructose-1,6-diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) (GINSBURG and MEHLER, 1966). By this binding one should expect changes in physical parameters of the enzyme. Indeed MUUS (1956) demonstrated that in the presence of chloride ions α-amylase showed changes in some physical properties (solubility and electrophoretic mobility). The same was observed for γ-glutamyl transpeptidase (glutamine: D-glutamyl glutamyl transferase, EC 2.3.2.1) from kidney bean fruit. GOORE and THOMPSON (1967) demonstrated by fluorescence spectroscopy that citrate changed the protein conformation of this enzyme in such a way that the active site was more accessible to substrate and inhibitors.

Dissociation into subunits or aggregation with a concomitant increase of activity can be another explanation of anion activation. Acetyl-CoA carboxylase (acetyl-CoA: carbon-dioxide ligase (ADP), EC 6.4.1.2) could be aggregated by preincubation with citrate anions at 30°. If the native enzyme was regarded as a monomer after preincubation a trimer was obtained. This aggregation was reversed by dilution of the citrate (VAGELOS, ALBERTS and MARTIN, 1962, 1963).

Nowadays it is known that a number of anions can function as allosteric effectors of enzymes. The formation of the enzyme-allosteric effector complex brings about a discrete reversible alteration of molecular structure (allosteric transition) which modifies the active site and changes one or several of the kinetic parameters characterizing the catalytic activity of the enzyme (MONOD, CHANGEUX and JACOB, 1963). For example, such an allosteric effect was found for the above-mentioned acetyl-CoA carboxylase. Citrate acted as an allosteric effector by changing the conformation of the enzyme (RYDER *et al.*, 1967).

Thus the effects of anions on the activity of enzymes can be attributed to: unspecific enhancement of the enzymic activity at the optimal pH, an effect on the enzyme-substrate complex, a binding of anions to the enzyme and conformational changes (*e.g.* allosteric effects).

The anion effects described here do not resemble those observed with allantoate amidohydrolase particularly: one of the most striking differences is that allantoate amidohydrolase was activated by the

anions, not at the pH optimum but at lower pH values, by preincubation. Furthermore the reversal of the activation by cations (7.1) already indicated that another mechanism was operative. The enzyme was activated when brought into contact with high salt molarities not only at pH 6 but also at the pH optimum: without preincubation a gradual increase of activity was observed, while preincubation abolished this lag period. At the first sight this seemed to be explained by an allosteric effect (MONOD, CHANGEUX and JACOB, 1963), but since the concentration of the salt was extremely high we do not believe this to be correct. More likely the high salt molarity will influence the charge distribution of the enzyme but the anions will not be bound direct to the enzyme.

Although much information has already been obtained about the activation of the enzyme allantoate amidohydrolase, it is still insufficient to design a mechanism which accounts for all the facts observed. In the next Chapter we shall discuss the phenomena of inactivation and stabilization and possibly all these effects together can give us a better insight into the mechanism of activation of this particular enzyme.

CHAPTER 7

INACTIVATION AND STABILIZATION OF ALLANTOATE AMIDOHYDROLASE

In the preceding Chapter we discussed that enzyme activation was probably due to a removal of incorrectly bound metal ions from the enzyme and that most likely Mn^{2+} ions were involved.

In earlier experiments (VOGELS, 1966) an inactivation (92 %) was observed when the enzyme, activated at pH 2.6 for 30 sec at 23°, was subsequently treated for 210 sec at the same temperature and a pH of about 6. Inactivation and activation are reversible processes; the inactivated enzyme could be activated again by a short pretreatment at low pH (VOGELS, 1966).

In contrast to this result in our experiments an activation took place at pH 6 (6.1.2). An important difference between the experiments at pH 6 performed by VOGELS (1966) and those described in 6.1.2 was the presence of EDTA in the preincubation mixtures of our experiments. Therefore, we assumed it to be possible that the inactivation of enzyme observed by VOGELS (1966) was due to metal ions which combined at pH 6 with the activated enzyme, thus yielding inactive enzyme. This seemed the more acceptable since we demonstrated the presence of Mn^{2+} ions in crude enzyme preparations by classic chemical methods and electron proton resonance spectroscopy. Furthermore in the citrate buffer used by VOGELS (1966) for activation at low pH Mn^{2+} ions were present (1.25×10^{-5} M final concentration during activation).

If this assumption were true then the reversibility of the activation and inactivation processes could be explained as a removal and binding of metal ions, respectively. Therefore, the first approach to gain a better insight into the mechanism of inactivation was made by investigating the effect of metal ions on the inactivation process.

7.1 INACTIVATION BY BIVALENT CATIONS

In Fig.38 the inactivation of allantoate amidohydrolase at pH 6 by several cations is shown as a function of time. The enzyme was inactivated most rapidly in the presence of Zn^{2+}. This effect of Zn^{2+} was not merely a result of inactivation of the enzyme, since Zn^{2+} ions strongly inhibited the enzymic reaction at the optimal pH (5.5). In contrast to Zn^{2+} ions, both Co^{2+} and Mn^{2+} stimulated the enzymic reaction (5.5) but in the presence of the same ions the enzyme was inactivated rapidly at pH 6. Results similar to those observed with Mn^{2+}

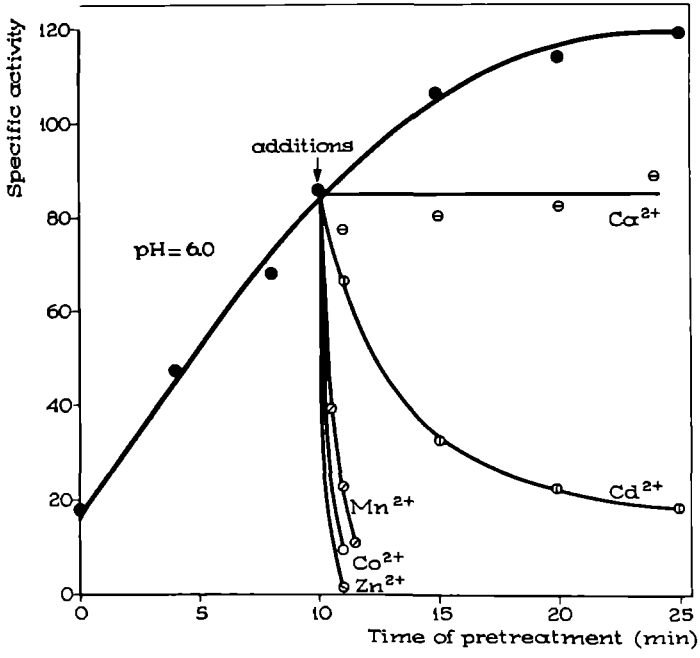


Figure 38

Effect of bivalent cations on the activity of allantoate amidohydrolase from *S.allantoicus* activated at pH 6.05. In order to activate 0.63 ml purified enzyme, containing 17 μg protein and 50 mμmoles EDTA in 0.05 M Tris-HCl buffer (pH 7.5), was mixed with 1.8 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.0). In the inactivation experiment 0.6 ml purified enzyme containing the same amount of protein and EDTA in buffer was mixed with 1.8 ml of the 0.1 M phosphate buffer. At t = 10 min of the preincubation period 0.03 ml water containing 0.46 μmole of the cation was added. At the indicated time intervals activity was tested as given in Fig.25.

and Co^{2+} at pH 6 were obtained with Cd^{2+}; this ion was less effective in the activity test (5.5) and also the inactivation at pH 6 was less rapid in the presence of this ion. Ca^{2+} ions, which possessed only a very weak cofactor activity (5.5), were nearly ineffective in inactivation of the enzyme at pH 6.

Table 13 represents the effect of cations, displayed at three pH values,

Table 13

Effect of bivalent cations on the activity of allantoate amidohydrolase from *S.allantoicus* activated at pH 6.05

0.46 ml purified enzyme solution containing 56 µg protein and 79.5 mµmoles EDTA in 0.05 M Tris-HCl buffer (pH 7.5) was treated for 30 min at 30° with 1.4 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.05). 0.2 ml of this mixture was added to 0.6 ml 0.1 M phosphate (pH 6.05), Tris-HCl (pH 7.9) or 0.13 M diethanolamine-HCl buffer (pH 8.5) and 0.01 ml of the cation solution. Final concentrations of the cation and EDTA were 10^{-4} M and 10^{-5} M, respectively. After 10 min treatment at 30° the enzyme activity of the pretreated mixture was tested by addition of 2 ml buffered substrate solution containing, per ml, 41.2 µmoles sodium allantoate, 5.9 µmoles GSH, 0.15 µmole $MnSO_4$ and 130 µmoles diethanolamine-HCl buffer (pH 8.8). The specific activity of the enzyme treated for 30 min at 30° and pH 6.05 was 103. This value was chosen as 100 %.

Cation (10^{-4}M)	Enzymic activity (%) after pretreatment of the enzyme for 10 min at 30°		
	pH 6.05	pH 7.45	pH 8.45
None	100	13	7
Ba^{2+}	98	15	5
Ca^{2+}	93	6	9
Mg^{2+}	93	26	9
Fe^{2+}	88	10	4
Pb^{2+}	108	108	53
Hg^{2+}	101	70	78
Cd^{2+}	52	106	73
Ni^{2+}	20	38	20
Co^{2+}	13	42	24
Mn^{2+}	5	10	100
Cu^{2+}	8	2	8
Zn^{2+}	4	4	3

on the activity of the enzyme activated at pH 6.05. In the absence of cations an inactivation occurred at pH 7.45 and pH 8.45; since this inactivation took place in the absence of bivalent cations and appeared to occur spontaneously we will call this phenomenon instability (2.5). Among the cations tested Ba^{2+}, Ca^{2+}, Mg^{2+} and Fe^{2+} had no or little effect at the pH values tested. Pb^{2+} and Hg^{2+} did not inactivate the enzyme but were fairly good stabilizers. Cd^{2+}, Ni^{2+} and Co^{2+} inactivated the enzyme at pH 6.05, but were moderate stabilizers at higher pH values. At pH 6 Cu^{2+} acted as 'inactivator' but at both other pH values it displayed a small or no effect. In this concentration Cu^{2+} did not inhibit the enzymic reaction. The inactivation by Zn^{2+} could be explained by the inhibiting effect of this ion on the enzymic reaction at the pH optimum (5.5). Mn^{2+} acted as an 'inactivator' at pH 6.05 but was the best stabilizer of all cations tested at pH 8.45. It appeared that two effects were operative: a) an inactivation of activated or already active enzyme molecules by cations and b) an instability of the active or activated enzyme at pH 7.45 and pH 8.45 as a result of dilution; this process could be counteracted by certain cations.

We shall discuss these two phenomena separately. Inactivation will be tested with Mn^{2+} ions as at pH 6 the strongest inactivating effect was observed in the presence of these cations (Table 13).

7.2 EFFECT OF pH ON THE INACTIVATION BY Mn^{2+} IONS

At pH 6 an inactivation of the enzyme by Mn^{2+} ions was observed. However, at pH values below 4.5 Mn^{2+} ions exerted no effect on the enzymic activity, since VOGELS (1966) observed the same specific activities when acid-activation was performed in the presence or absence of Mn^{2+} ions. Also at pH 8.45 no inactivating effect of Mn^{2+} ions was observed. Therefore, the inactivation by Mn^{2+} was investigated more extensively.

We could confirm the results obtained by VOGELS (1966). No effect on the rate and plateau of activation was observed when in the pretreatment mixture given in Fig.25 Mn^{2+} ions (10^{-4}M) were present. At higher pH values, up to pH 5.3, Mn^{2+} ions (10^{-4} M) exhibited also no

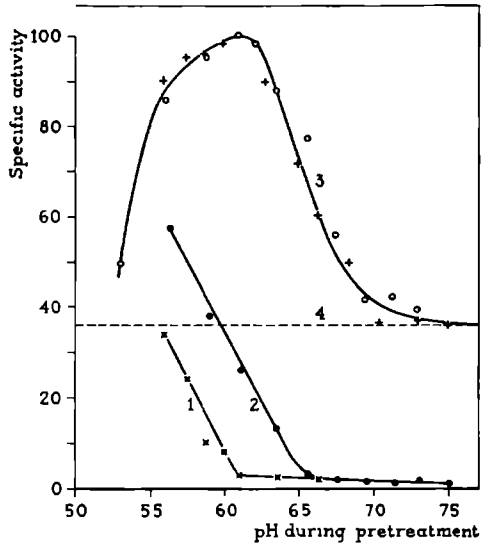


Figure 39

Effect of manganous ions, present during activation, on the specific activity of allantoate amidohydrolase. Activation and activity tests were performed as given in Fig.32. In the experiments represented by curves 1 and 2 $MnSO_4$ was added at the start of the activation period (final concentration 1.9×10^{-4}M). Curve 1 was obtained by using 0.1 M KH_2PO_4-Na_2HPO_4 buffer and curve 2 by using 0.1 M citric acid-Na_2HPO_4 buffer. Curve 3 represents the activation curve in the absence of added Mn^{2+} ions and curve 4 the activity of the untreated enzyme material.

effect on the extent and rate of activation. These experiments were performed as given in Fig.32. However, at pH values above 5.3 a lower extent of activation was obtained in the presence of added Mn^{2+} than in its absence (Fig.39). The specific activity of the enzyme material used in these experiments was 36.4 when tested without pretreatment. Therefore, not only a decrease of the extent of activation occurred in the presence of Mn^{2+}, but also an inactivation of enzyme molecules which were already active took place.

The extent of inactivation by Mn^{2+} ions was dependent on the buffer anion used (Fig.39). In phosphate buffers the inactivation was more pronounced than in citric acid-phosphate buffers. Fig.40 shows that the rate of inactivation is different in the two buffers. It appeared that the higher inactivation rate was obtained in those systems which had

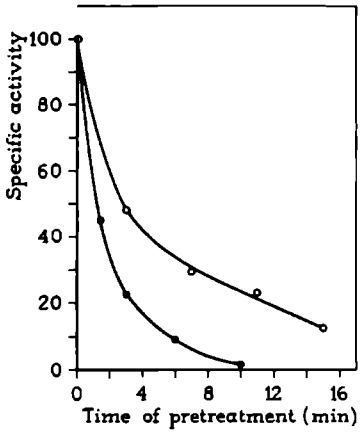


Figure 40

Inactivation of allantoate amidohydrolase at pH 6.1 by Mn^{2+} ions in the presence of phosphate (•) or citric acid-phosphate (o) buffer. Activation was performed as given in Fig.32. Inactivation was started by the addition of $MnSO_4$ to the activation mixture. The final Mn^{2+} concentration was 2×10^{-4}M. Activity was tested by the addition of a 0.2 ml aliquot of the inactivation mixture to 2 ml substrate solution. The final composition, per ml, was 31.2 μmoles sodium allantoate, 4.28 μmoles GSH, 0.11 μmole $MnSO_4$, 118 μmoles diethanolamine-HCl buffer (pH 8.8) and 3.1 μg enzyme protein.

the smaller complexing ability and thereby the higher concentration of free Mn^{2+} in solution. A similar effect was observed when phosphate buffers with different molarities were used.

Enzyme, activated at pH below 4, was inactivated by a subsequent treatment at a pH of about 6 (VOGELS, 1966). It was demonstrated that addition of Mn^{2+} ions at pH 6 to the enzyme, activated at the same pH, brought about a rapid inactivation of the activated enzyme (7.1). Since in most of the experiments performed by VOGELS (1966) Mn^{2+} ions were present during the activation treatment at low pH, the inactivation observed at pH 6 could be explained by the presence of this ion during the treatment at this pH. We repeated and extended these experiments by treating acid-activated enzyme at various pH values (Fig.41). In the absence of added Mn^{2+} ions and EDTA the acid-activated enzyme was rather stable below pH 4.5 and above pH 8. Above pH 4.5 an inactivation occurred and maximal inactivation was obtained at pH 6. This in-

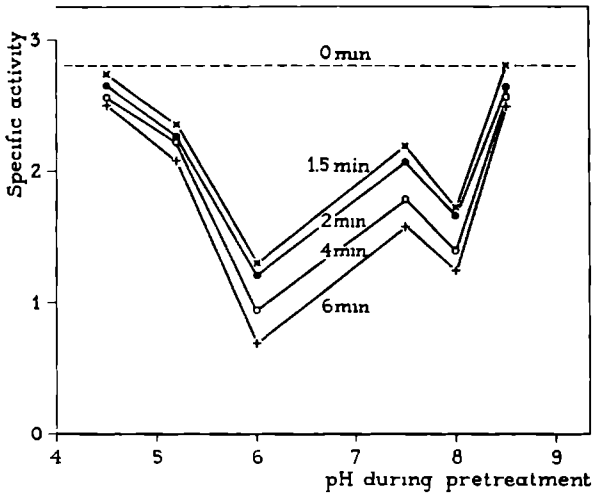


Figure 41

Effect of treatment at various pH values on the activity of allantoate amidohydrolase from *S.allantoicus* activated at pH 3.83. 1 vol. dialyzed cell-free extract (5.16 mg protein in 0.05 M Tris-HCl buffer, pH 7.5) was treated for 40 sec at 0^{o} with 3 vol. 0.05 M sodium citrate-HCl buffer (pH 3.0). The resulting pH of the mixture was 3.83. After activation a 0.4 ml aliquot of the mixture was added to 1.2 ml of buffer. Buffers used were 0.1 M sodium acetate-acetic acid (pH 4.5-5.2), 0.1 M KH_2PO_4-Na_2HPO_4 (pH 6), 0.05 M Tris-HCl (pH 7.5-8.0) and 0.1 M diethanolamine-HCl (pH 8.5). Activity was tested by the addition of a 0.2 ml aliquot of the mixtures to 2 ml substrate solution with the composition given in Fig.25 for curve 1.

activation was probably due to a release of Mn^{2+} ions from the enzyme and other molecules by the pretreatment at low pH. These ions could combine again with the activated enzyme at certain pH values, thereby causing inactivation. The rate of inactivation was highest at a pH of about 6. The effects at pH values between 7.5 and 8.5 will be discussed later (7.3).

7.2.1 *Effect of allantoate and glutathione on the rate of inactivation by Mn^{2+} ions at pH 6.05*

The presence of substrate during inactivation by Mn^{2+} ions at pH 6.05 inhibited the inactivation appreciably (Fig.42). The effect of GSH was almost negligible. It was already demonstrated that allantoate inhibited

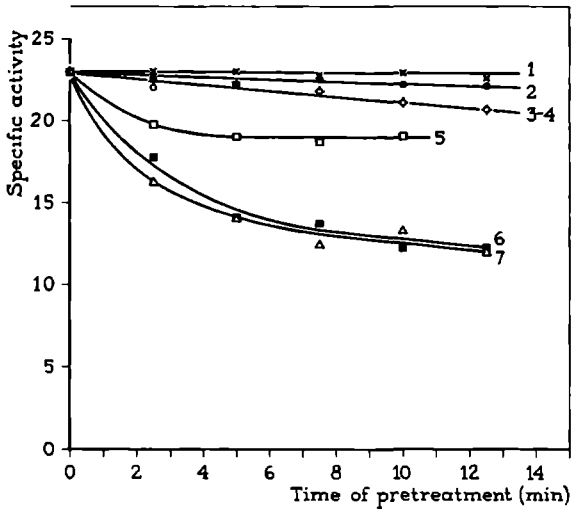


Figure 42

Effects of allantoate and GSH on the rate of allantoate amidohydrolase inactivation by Mn^{2+} ions at pH 6.05. 0.25 ml purified enzyme (98 µg protein and 42.5 mµmoles EDTA) was treated for 30 min at 30° with 0.75 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.95). At this time the activation mixtures were supplemented with 0.2 ml aliquots containing the following substances, expressed in final concentration in the activated mixtures: (1) no addition; (2) 2 x 10^{-2}M sodium allantoate; (3) 2 x 10^{-2}M GSH; (4) water; (5) 2 x 10^{-2}M sodium allantoate and 1.5 x 10^{-5}M $MnSO_4$; (6) 2 x 10^{-2}M GSH and 1.5 x 10^{-5}M $MnSO_4$ and (7) 1.5 x 10^{-5}M $MnSO_4$. Activity was tested at the indicated time intervals by addition of a 0.2 ml aliquot of the final mixtures to 2 ml substrate solution. The assay mixtures contained finally, per ml, 29.2 µmoles sodium allantoate, 4.23 µmoles GSH, 0.14 µmole $MnSO_4$, 118 µmoles diethanolamine-HCl buffer (pH 8.8) and 3.2 µg protein.

the activation of the enzyme at pH 6 (6.5). These experiments indicate that Mn^{2+} ions are bound at or very near at the active center of the enzyme. Formation of the enzyme-substrate complex possibly inhibits Mn^{2+} ions to gain access to their binding place at the active center. Thus, substrate will protect active enzyme at pH 6 against inactivation by Mn^{2+}.

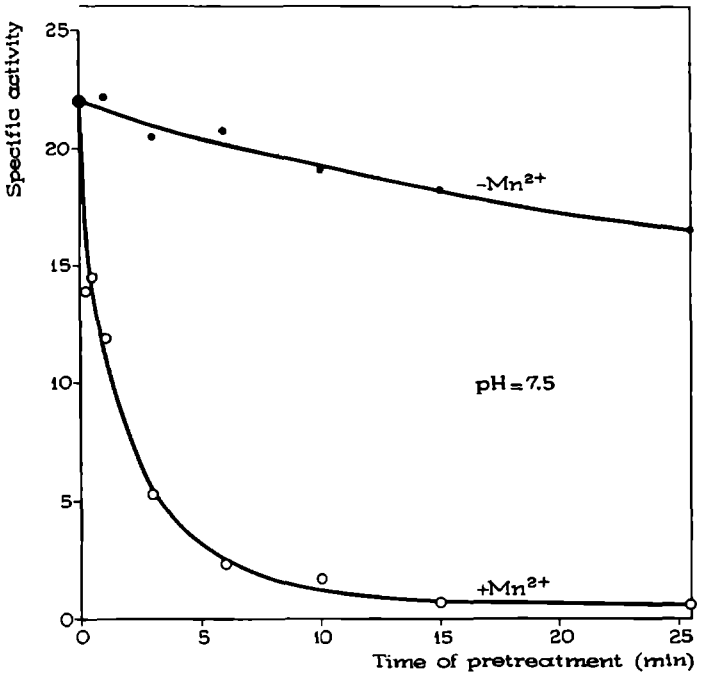


Figure 43

Effect of pretreatment at pH 7.5 on the activity of allantoate amidohydrolase in the presence and absence of Mn^{2+}. 0.63 ml purified enzyme (17 µg protein and 0.1 µmole EDTA) was mixed with 1.8 ml 0.05 M Tris-HCl buffer (pH 7.5). In one sample Mn^{2+} was present in a concentration of 1.9×10^{-4}M. Activity was tested by addition of a 0.2 ml aliquot of the pretreated mixture to 2 ml substrate solution which contained, per ml, 23 µmoles sodium allantoate, 5.9 µmoles GSH, 0.15 µmole $MnSO_4$ and 130 µmoles diethanolamine-HCl buffer (pH 8.8).

7.3 STABILITY OF THE ENZYME

It followed from Table 13 (7.1) that enzyme which was activated at pH 6 and subsequently treated at pH 7.45 or 8.45 lost most of its activity. Furthermore this table revealed that Mn^{2+} ions protected the active enzyme against a decrease of activity at pH 8.45. These effects could not be understood by assuming a simple binding of bivalent cations to the enzyme stripped of these ions by previous activation. We studied these effects more in detail at pH 7.5 and pH 8.5 to gain more insight into the mechanisms of inactivation and stabilization.

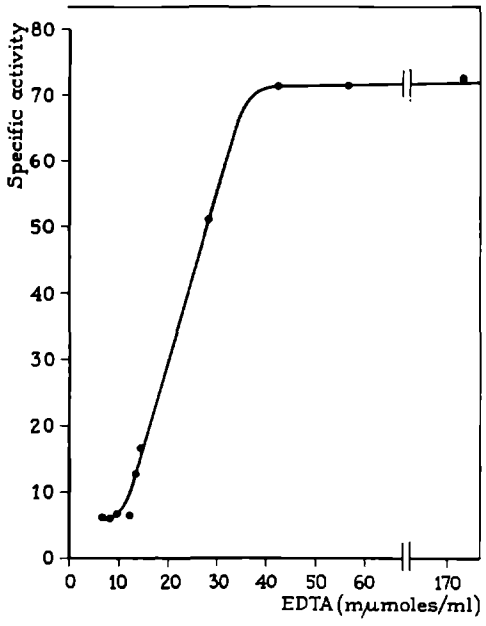


Figure 44

Effect of EDTA concentration on the activity of allantoate amidohydrolase during pretreatment at pH 7.5. 0.1 ml purified enzyme solution (13 μg protein and 17 mμmoles EDTA) was diluted with 1 ml Tris-HCl buffer (pH 7.5) containing varying amounts of EDTA. The amount, per ml, of EDTA in the pretreatment mixtures is given in the figure. After treatment for 10 min at 0^{o} a 0.2 ml aliquot of the mixture was added to 2 ml substrate solution, which had the same composition as given in Fig.43.

7.3.1 *Stability of the enzyme at pH 7.5*

Fig.43 shows the effect of pretreatment of the enzyme at pH 7.5 in the presence and absence of Mn^{2+}. Inactivation occurred in the presence of Mn^{2+} as expected from previous results (7.1). In the absence of Mn^{2+} ions also a slight decrease of enzymic activity occurred, which according to the definitions given (2.5) will be due to instability. However, comparison to Table 13 evinced the enzyme to be much more stable in the experiment of Fig.43. The concentration of EDTA was different in the two experiments, *viz.* 1.1×10^{-5}M and 4.1×10^{-5}M in the experiments of Table 13 and Fig.43, respectively. Therefore, the instability of the enzyme at pH 7.5 was possibly correlated to the concentration of EDTA in the mixture. Fig.44 shows the effect of the EDTA con-

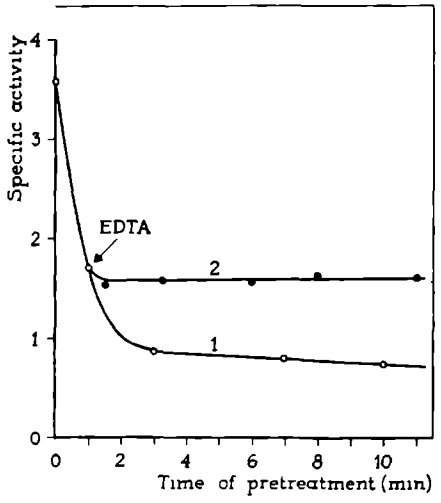


Figure 45

Decrease of activity of allantoate amidohydrolase from crude cell-free extract by dilution with EDTA-free buffer at pH 7.5; effect of EDTA addition. 0.4 ml cell-free extract (1.42 mg protein and 68 mμmoles EDTA) was treated for 30 min at 30° with 1.2 ml 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 5.95). 0.4 ml of the activation mixture was added to 2 ml 0.05 M Tris-HCl buffer; the pH of the mixture was 7.5. For curve 2 EDTA was added at t = 1 min to a final concentration of 10^{-4}M. Activity was tested by the addition of 0.2 ml aliquots of these mixtures to 2 ml substrate solution, which had the composition as given in Fig.43.

centration during pretreatment of the enzyme at pH 7.5. On lowering the EDTA concentration beneath 4×10^{-5}M a very sharp decrease of enzymic activity occurred, *viz.* at least 4×10^{-5}M EDTA was necessary for the stability of the active enzyme at pH 7.5. A small residual activity remained even in the absence of EDTA. Similar effects were obtained on dilution with Tris, phosphate, triethanolamine and diethanolamine buffer or quartz-distilled water. Instability could be prevented in all cases by supplementing the diluting buffer or the water with 10^{-4}M EDTA.

Instability of the enzyme at pH 7.5 was observed with activated and already active untreated enzyme and with the enzyme from crude cell-free extracts. In all cases a concentration of 10^{-4}M EDTA was able to stabilize the enzyme. Addition of EDTA to a final concentration of 10^{-4} M at a certain time to the diluted enzyme solution at pH 7.5 almost immediately stopped the decrease of enzymic activity (Fig.45).

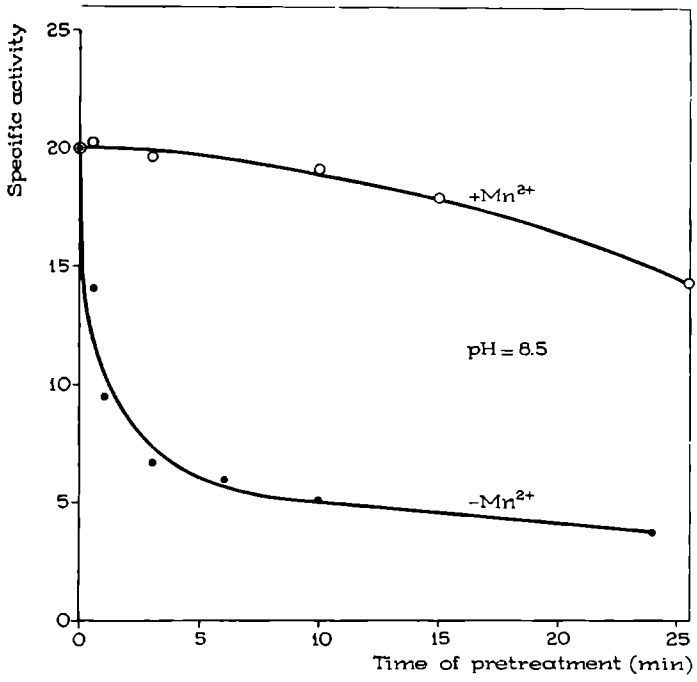


Figure 46

Effect of pretreatment at pH 8.5 on the activity of allantoate amidohydrolase in the presence and absence of Mn^{2+}. The same procedure was followed as given in Fig.43, except that the 0.05 M Tris-HCl buffer (pH 7.5) was replaced by 0.13 M diethanolamine-HCl buffer (pH 8.5).

It can be concluded that at pH 7.5 two effects appeared to be responsible for the observed decrease of enzymic activity: 1) inactivation by metal ions (7.1) and 2) a decreased stability; this decrease could be counteracted by EDTA (7.3) and by certain metal ions (7.1). Of course EDTA will also exert an effect on the inactivation by metal ions by competition with the enzyme for the cation.

7.3.2 *Stability of the enzyme at pH 8.5*

The effect of pretreatment of the enzyme at pH 8.5 in the presence and absence of Mn^{2+} is shown in Fig.46. In accordance with the result obtained in Table 13 (7.1) Mn^{2+} ions protected the enzyme against the decrease of activity observed in the absence of these ions. A similar

effect was observed with other cations (Table 13, 7.1). Addition of EDTA (10^{-4}M) also stabilized the enzyme almost completely. A similar phenomenon was observed at pH 7.5 (7.3.1). Dilution of the enzyme preparation also decreased the enzymic activity at pH 8.5, but addition of EDTA to the dilution buffer did protect the enzyme against this instability and the extent of protection was dependent on the EDTA concentration.

7.3.3 *Stability as a function of pH*

From 7.3.1 and 7.3.2 it was clear that under certain conditions the enzyme was unstable. Mn^{2+} and EDTA were able to protect the enzyme against this instability at pH 8.5, but at pH 7.5 only EDTA could stabilize the enzyme, while Mn^{2+} acted as an 'inactivator' of the enzyme. Since there was a clear-cut difference between the effects at pH 7.5 and 8.5 the stability and instability under different conditions were studied in the pH range 7.5-11 (Fig.47). Only a small residual specific activity was measured at all pH values (curve 1) when enzyme was tested which was previously dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) to remove EDTA. This low specific activity could be expected because it was already known that EDTA affected the stability at pH 7.5 (7.3.1). The effect of EDTA on the stability was studied at that concentration (4.25×10^{-5}M) which resulted in stabilization at pH 7.5, but yielded a less stable enzyme at pH 8.5. Above pH 7.5 (curve 2) a rapid decrease of the enzymic activity took place and under the conditions tested the highest diminution of activity occurred at pH 8. On increase of the pH at values above 8 the enzyme appeared to be more stable in the presence of this low EDTA concentration. At a pH of about 10.5 the enzyme was almost completely stable. When a higher EDTA concentration (2.5×10^{-4}M) was present during pretreatment at pH values above 7.5 only a small decrease of specific activity occurred (curve 4). If both Mn^{2+} (10^{-4}M) and EDTA (4.25×10^{-5}M) were present during the pretreatment period (curve 3) the inactivation by Mn^{2+} ions observed at pH 7.5 diminished at increasing pH. At pH values above 8 no inactivation was observed and a stabilization by Mn^{2+} ions took place.

The enzyme is unstable in the absence of EDTA and Mn^{2+} or in the

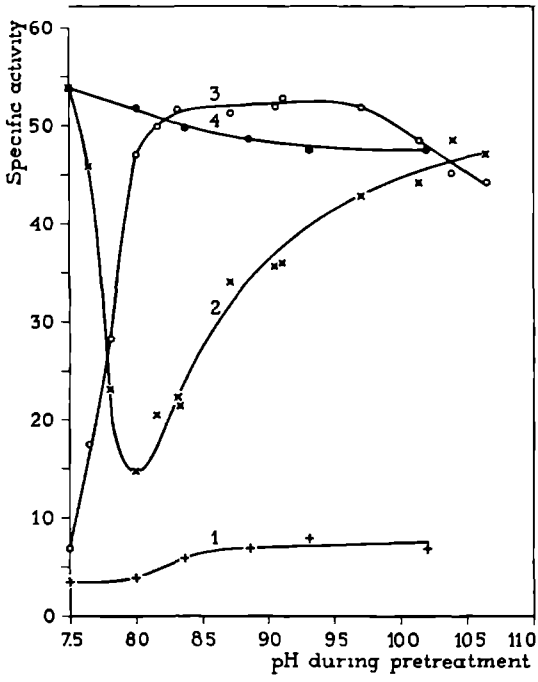


Figure 47

Stability of allantoate amidohydrolase during pretreatment at basic pH values; effects of Mn^{2+} and EDTA. Purified enzyme (specific activity 56.5) was pretreated for 5 min at 30° at various pH values (total volume was 0.8 ml). The mixtures contained, per ml, 15.8 µg protein (for curve 1 dialyzed EDTA-free enzyme was used), 7.5 x 10^{-2}M buffer anion and furthermore 4.25 x 10^{-5}M EDTA (2) or 4.25 x 10^{-5}M EDTA + 10^{-4}M Mn^{2+} (3) or 2.5 x 10^{-4}M EDTA (4). Activity was tested by the addition of 2 ml substrate solution which contained, per ml, 41.4 µmoles sodium allantoate, 5.9 µmoles GSH, 0.15 µmole $MnSO_4$ and 130 µmoles diethanolamine-HCl buffer (pH 8.8). Buffer anions used during pretreatment were Tris (pH 7.5-8.4), diethanolamine (pH 8.3-9.2) and bicarbonate (pH 9.1-10.7).

presence of a low EDTA concentration. Highest instability was observed at pH 8. At pH values above 8 both EDTA and Mn^{2+} stabilize; at pH values below 8 EDTA stabilizes and Mn^{2+} inactivates the enzyme.

After a complete activation at pH 6.05 the specific activity decreased on lowering the EDTA concentration (Fig.48). The extent of the decrease of specific activity was not proportional to the extent of the dilution. Therefore, probably also in this case a critical amount of EDTA was necessary to stabilize the enzyme.

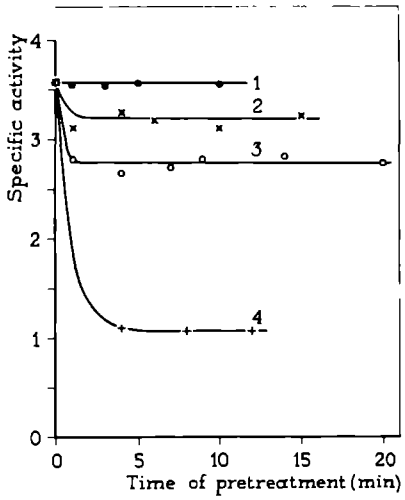


Figure 48

Influence of dilution at pH 6.05 of allantoate amidohydrolase activated at pH 6.05 with phosphate buffer. Activation was performed as given in Fig.45. 0.5 ml aliquots of the activation mixture were added to (1) 2.5 ml 0.1 M phosphate buffer (pH 6.05) containing 2.2 x 10^{-4}M EDTA, (2) 2 ml 0.1 M phosphate buffer (pH 6.05), (3) 2.5 ml 0.1 M phosphate buffer (pH 6.05) or (4) 4.5 ml 0.1 M phosphate buffer (pH 6.05). Activity was tested at the indicated time intervals as given in Fig.43.

7.3.4 *Effect of the time of pretreatment on the stability of allantoate amidohydrolase*

In the preceding paragraphs (7.3.2 and 7.3.3) it was noticed that a decrease of specific activity of activated or active enzyme occurred when the EDTA concentration was lowered. At first a rapid decrease occurred, followed by a slower one till an apparent equilibrium was established between the active and inactive enzyme molecules. To study the effect of the pretreatment period on the stability of allantoate amidohydrolase a pH of 8.0 was chosen, since at this pH the active enzyme was unstable in the presence of a low EDTA concentration (4.25 x 10^{-5}M) but fairly good stabilized by Mn^{2+}ions (10^{-4}M; Fig.47). The result is given in Fig.49. It appeared that both Mn^{2+} and EDTA (curves 3 and 4) were able to stabilize the enzyme against the decrease of specific activity obtained in the absence of these compounds (curve 2). A complete removal of EDTA by extensive dialysis

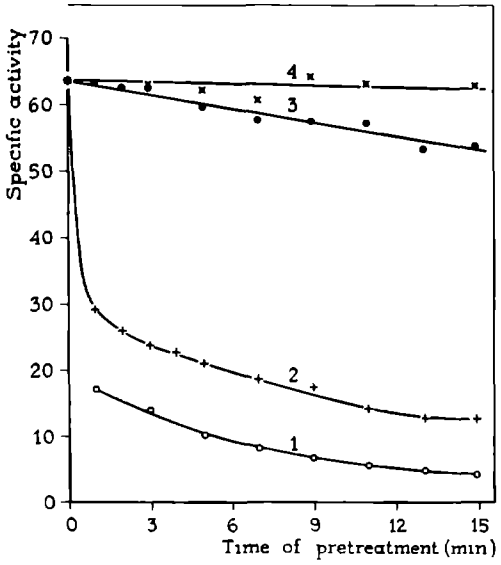


Figure 49

Effect of the time of pretreatment at pH 8.0 on the stability of allantoate amidohydrolase in the absence or presence of Mn^{2+} and EDTA. 1 Vol. purified enzyme solution was mixed with 3 vol. 0.1 M Tris-HCl buffer (pH 8.0). The pretreatment mixture at 30° contained, per ml, 10.8 µg protein (for curve 1 dialyzed EDTA-free enzyme was used), 87.5 µmoles Tris-HCl buffer (pH 8.0) and furthermore 4.2 x 10^{-5}M EDTA (2) or 4.2 x 10^{-5}M EDTA and 10^{-4}M Mn^{2+} (3) or 2.3 x 10^{-4}M EDTA (4). Activity was tested as given in Fig. 47.

yielded still lower specific activities. Just like at pH 8.5 (7.3.2) a rapid diminution of activity took place at first (about 2 min) followed by a further gradual decrease, finally resulting in an apparent equilibrium between active and inactive enzyme molecules.

During the present study the specific activity of the untreated allantoate amidohydrolase from cell-free extracts varied between 0.1 and 1 unit per mg protein. This was about 10 times higher than the values obtained by VOGELS (1966). This author prepared cell-free extracts in the absence of EDTA; since EDTA is necessary for the stability of the active enzyme, it is evident that the specific activities of his enzyme preparations will be lower than those measured by us.

7.4 EFFECT OF ALLANTOATE AND GLUTATHIONE ON THE STABILITY AT pH 8.5

Treatment of the purified enzyme at pH 8.5 in the presence of low EDTA concentrations resulted in a decrease of enzymic activity (Table 13). Without any addition only 7 % activity was found after a treatment for 10 min at 30^{o}. If allantoate (2×10^{-2}M) was present during the treatment at this pH 15 % of the original activity remained. The presence of GSH (1.5×10^{-2}M) resulted in a remaining activity of about 50 %. Both GSH and allantoate could stabilize the enzyme at pH 8.5; GSH was a fairly good stabilizer but the substrate did protect only to a small extent against the instability.

7.5 CORRELATION BETWEEN INACTIVATION AND INSTABILITY

The establishment of an equilibrium between active and inactive enzyme observed both during activation (Fig.33a) and in the experiments on the stability (Figs.46-49) suggested a correlation between both processes, *viz.* instability might be due to an inactivation of active or activated enzyme by cations. The reversibility of the processes of inactivation and instability also indicated this correlation; this reversibility will be discussed in the next section (7.6).

The establishment of an equilibrium between active and inactive enzyme molecules was shown to be dependent on several factors (6.4.2); one of these was the concentration of free Mn^{2+} ions present during the activation process. A shift in the equilibrium towards the inactive enzyme molecules could be brought about by the addition of Mn^{2+}; these ions were able to inactivate active or activated enzyme below pH 8 (7.1). An inactivation or instability of the active enzyme was observed in mixtures containing a low concentration of EDTA. This effect was observed at pH 6 (7.3.3), pH 7.5 (7.3.1) and pH 8.5 (7.3.2). A protection against this decrease of activity was obtained when the concentration of complexing agents, *e.g.* EDTA, was enhanced (Fig. 47, curve 4). For example in the absence of complexing compounds or in the presence of 1,10-phenanthroline (2×10^{-4}M), citrate (7.5×10^{-2}M) and EDTA (2 x

10^{-4}M) 70% or 29%, 27% and 14% decrease of the enzymic activity was observed, respectively, after 10 min at pH 8.5.

A distinction can be made between the effects observed below and above pH 8. Below pH 8 and above pH 5 Mn^{2+} ions inactivate the enzyme; the inactivation was thought due to binding of Mn^{2+} ions to certain ligands in the active center, which resulted in the catalytically inactive configuration of the enzyme. From this viewpoint seen, stabilization by EDTA below pH 8 will consist in a prevention of the inactivation by Mn^{2+} ions present in the free state in the pretreatment mixture. These Mn^{2+} ions may be contaminations in the solutions used or may dissociate from the active enzyme molecules. Such a dissociation will be accelerated in dilute solutions; the same is true for the Mn^{2+} - EDTA complex.

Above pH 8 both complexing substances and Mn^{2+} can stabilize the enzyme. In the presence of these ions the active configuration of the enzyme is obtained above pH 8 and further maintained. Presumably at a pH of about 8 there is a change in the charge distribution in the active center and other ligands are involved in binding of Mn^{2+} below pH 8 as compared to pH values above 8. In the presence of an excess of EDTA no enzymic activity was measured. Addition of an excess of Mn^{2+} ions to such a mixture fully restored the activity. Therefore, it appeared that Mn^{2+} is rather loosely bound in the active enzyme. The enzyme is rather unstable at pH values above 8, which indicates that the ligands involved in binding of Mn^{2+} ions in the inactive enzyme still bind Mn^{2+} at pH values above 8. The bond with these ligands appeared much stronger since EDTA cannot remove Mn^{2+} easily from these ligands and thus activate the enzyme. Loss of activity of the enzyme as a result of instability at pH 8.5 could only be restored by activation and not by stimulation of the enzyme (7.6).

Stabilization by EDTA at pH values above 8 consequently can be regarded as binding of Mn^{2+} ions which had dissociated from the active enzyme. Stabilization by Mn^{2+} cannot be explained in such a way; presumably Mn^{2+} ions shield those ligands which are involved in the 'faulty' binding of Mn^{2+} and thereby prevent an intramolecular rearrangement by which Mn^{2+} bound in the active enzyme is transferred to the 'faulty' ligands.

7.6 REVERSIBILITY OF ACTIVATION AND INACTIVATION

VOGELS (1966) demonstrated that the enzyme, activated for 30 sec at 0^{o} and pH 2.6, could be reversibly inactivated at a pH of about 6. In most of his experiments Mn^{2+} ions were present and it was shown by us (7.1) that inactivation of active enzyme could be achieved by these ions. The activation and inactivation reaction can be represented by the following equation:

$$E^{a}\text{-}Mn^{2+} + aH^{+} \rightleftharpoons EH_{a} + Mn^{2+} \qquad \text{(eq.1; 6.4.2.1)}$$

inactive enzyme — activated enzyme

Since both activation and inactivation could take place at pH 6 (6.1.2) it must be possible to reverse the activation effect at this pH. This was already shown in 7.1: enzyme, completely activated at pH 6.05 in the presence of phosphate anions and EDTA, could be inactivated again by the addition of Mn^{2+} (Table 13). In Table 14 the reversibi-

Table 14

Reversibility of activation and inactivation

For detailed information on the procedure followed, see 7.7.1.

Treatment	Total units	Specific activity
1 Purified enzyme (starting material)		
Untreated	35.4	68
Activated at pH 6 for 30 min at 30^{o} with 7.5 x 10^{-2}M phosphate and 4.25 x 10^{-5}M EDTA	52	100
2a Dialysis of untreated enzyme (16 h against Tris-HCl, pH 7.5; 0.01 M) and thereafter lyophilized	10	19
b Activation at pH 6	47.7	92
c Inactivation at pH 6 by 4 x 10^{-4}M $MnSO_4$ for 10 min at 30^{o}	3.3	6.3
d Solution adjusted to pH 8.6, Sephadex G-25 gel filtration, followed by activation at pH 6	45.4	87

lity of the activation and inactivation process is also shown. It appeared that both the decrease of specific activity by removal of EDTA (step 2a) and the inactivation by Mn^{2+} ions (step 2c) could be reversed by repeating the activation procedure at pH 6 in the presence of phosphate and EDTA. A similar reversibility was observed when enzyme, activated at pH 6 in the presence of 7.5×10^{-2}M oxalate and 4.25×10^{-5}M EDTA (specific activity 19), was inactivated at pH 6 by treatment for 10 min at 30° with 2×10^{-4}M $CuSO_4$ (specific activity 1.6) and subsequently treated for 10 min at 30° with 7.5×10^{-2}M oxalate and 4.25×10^{-5}M EDTA at pH 6 (specific activity 12.5). Therefore, we believe that the metal interactions are of a similar nature.

The question arose whether the decrease of activity due to instability of the enzyme (7.3) was a reversible or irreversible process. Fully activated enzyme was diluted with EDTA-free buffer at pH values at or above 7.5 and thereby the enzymic activity decreased. This material could be activated again up to the original specific activity by treatment at pH 6 for 30 min at 30° in the presence of 7.5×10^{-2}M phosphate and 4.25×10^{-5}M EDTA. No detectable activation of this material was obtained at or above pH 7.5 by the addition of EDTA (10^{-4}M) although further decrease of specific activity was stopped almost immediately by this addition (Fig.45). Activation, however, could occur at pH 7.5 or even at higher pH values (6.4.2, Fig.33a) in the presence of oxalate (7.5×10^{-2}M) and EDTA (4.25×10^{-5}M). From these results it followed that enzyme inactivated by Mn^{2+} ions and enzyme which had lost activity as a result of instability could be reactivated by the same procedure.

7.7 SPECIFIC ROLE OF Mn^{2+} IN THE PHENOMENA OF ACTIVATION, INACTIVATION, STABILIZATION AND STIMULATION

The enzyme allantoate amidohydrolase absolutely required a bivalent metal ion for catalytic activity; Mn^{2+} was the best cofactor (5.5). Inactivation of the enzyme at pH 6 by cations was most rapidly with Mn^{2+} (Table 13) and furthermore above pH 8 Mn^{2+} acted as the best metal ion stabilizer (Table 13).

In purified enzyme preparations the qualitative presence of Mn^{2+} was shown by means of the formaldoxime method of BARTLEY, NORTON and WERKHEISER (1957) (VAN DER DRIFT and VOGELS, 1967).

These facts together suggested that Mn^{2+} was probably the physiologically important cation for this enzyme, although participation of other cations could not be excluded completely. Therefore, we prepared the inactive Mn^{2+}-enzyme complex and determined the amount of bound Mn^{2+}.

7.7.1 *Preparation of the inactive Mn^{2+}-enzyme complex. Determination of bound Mn^{2+}*

Method A: 4 ml purified enzyme solution (52 U, 520 µg protein; specific activity of the untreated enzyme 68; specific activity 100, if fully activated; see also Table 14) was dialyzed against 2 l 0.01 M Tris-HCl buffer (pH 7.5) for 16 h to remove EDTA, present in the enzyme solution in a concentration of 1.7×10^{-4}M. The solution was lyophilized and the residue was taken up in 0.5 ml Tris-HCl buffer (0.05 M; pH 7.5) containing 1.7×10^{-4}M EDTA and activated for 30 min at 30° with 1.5 ml 0.1 M phosphate buffer (pH 6.0). After this treatment the activity of the enzyme was 47.7 U. Active enzyme molecules were inactivated by treatment with 0.1 ml $^{54}Mn^{2+}$ - $^{55}Mn^{2+}$ (final concentration 4×10^{-4}M) for 10 min at 30°. An enzyme activity test showed that after inactivation about 6.5 % (3.3 U) of the activity remained. The pH of the inactive enzyme solution was adjusted to 8.6 by addition of 0.6 ml 0.4 M diethanolamine. This solution was applied to a Sephadex G-25 column (68 cm x 1.8 cm) and the inactive enzyme was eluted with 0.05 M diethanolamine-HCl buffer (pH 8.6). Fractions (2 ml) were collected and tested for radioactivity and for enzymic activity after reactivation at pH 6 in the presence of 7.5×10^{-2}M phosphate and 4.25×10^{-5}M EDTA (Fig.50). No appreciable activity was measured when the enzyme was not reactivated. After reactivation 45.4 U were obtained; this was a recovery of 87.5 % over the whole procedure.

From the values given in Fig.50 the ratio Mn^{2+} *vs.* protein was calculated. During the inactivation process 20 µl $^{54}Mn^{2+}$ was present; in control tubes we measured that these 20 µl yielded 1.38×10^6 counts/100 sec. The amount of Mn^{2+} present during the inactivation

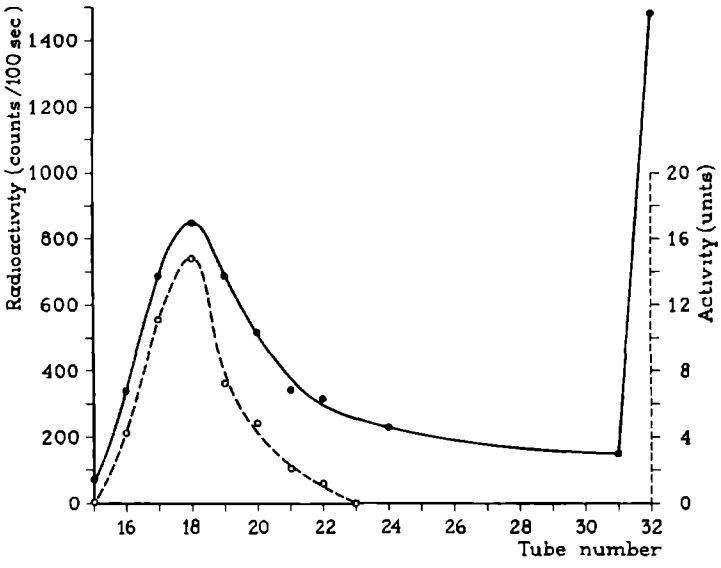


Figure 50

Gel filtration of $^{54}Mn^{2+}$-allantoate amidohydrolase on Sephadex G-25. Determination of radioactivity (2.2) and enzymic activity in the fractions collected. Enzymic activity was tested, after activation at pH 6, as given in Fig.47.

process was 0.84 μmole. Thus, 1 μmole Mn^{2+} will give $1.38 \times 10^6/0.84 = 1.64 \times 10^6$ counts/100 sec. The total radioactivity measured in the first peak of Fig.50 was 3748 counts/100 sec. This corresponded with $3748/1.64 \times 10^6 = 2.28 \times 10^{-3}$ μmole Mn^{2+}. The amount of protein present in the same tubes was 455 μg. The molecular weight of the enzyme was about 67 000 (VOGELS, 1966), thus 6.8×10^{-3} μmole of protein was present. This would mean that the ratio of moles of Mn^{2+} *vs.* moles of protein was $\frac{2.28 \times 10^{-3}}{6.8 \times 10^{-3}} = 1/3$. This value was not corrected for the 6.5 % enzyme molecules which remained in the active configuration after inactivation with Mn^{2+}. This ratio seemed to be surprisingly low, but we have to emphasize that possibly the conditions employed in the experiment are not optimal. Especially during gel filtration there might have been a great loss of radioactivity. A similar observation was made by

SCHLESINGER (1966) for the $^{65}Zn^{2+}$ - alkaline phosphatase complex (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1); after gel filtration on Sephadex G-25 only 10 % of the radioactivity remained. A similar effect was observed with acetyl coenzyme A synthetase (acetate : CoA ligase (AMP), EC 6.2.1.1) : one of the two bound $^{63}Ni^{2+}$ ions was easily removed by gel filtration, the other was not. No loss of radioactivity was observed on dialysis. Two different binding sites with different affinities for the metal ion were proposed (WEBSTER, 1967).

The only conclusion which we may draw from this experiment is that Mn^{2+} can be bound to activated enzyme molecules during the inactivation, which is due to the formation of a catalytically inactive Mn^{2+}-enzyme complex.

Method B: Determination of the amount of bound Mn^{2+} was also performed by means of the EPR technique. Purified enzyme (1.2 ml), containing 1.7 x 10^{-4}M EDTA and 144 µg of protein in 0.05 M Tris-HCl buffer (pH 7.5), was activated for 30 min at 30° with 3.6 ml of 0.1 M phosphate buffer (pH 6.0). This enzyme solution contained 11.7 U per ml. Subsequent inactivation in 6 x 10^{-4}M Mn^{2+} was performed as described in method A. After inactivation 0.9 U per ml was present. After extensive dialysis against 6 changes of 2 l 0.01 M Tris-HCl buffer (pH 7.5), from which the first four contained 1.7 x 10^{-4}M EDTA, the solution was lyophilized and taken up in 1 ml of 0.05 M Tris-HCl buffer (pH 7.5). Half of this solution was acidified by addition of 0.05 ml of 2 N HCl. Under these conditions all Mn^{2+} was assumed to be present in the solution as free Mn^{2+}. This concentration of free Mn^{2+} was measured from the intensity of the EPR spectrum and by comparison with a standard amount of Mn^{2+}. Since the Mn^{2+} concentration was very low we used a time-averaging computer (2.2). To the other half of the Mn^{2+}-enzyme complex 0.05 ml of quartz-distilled water was added and the solution was measured directly for free Mn^{2+} under the same conditions. By subtraction of the latter value (0.75 x 10^{-6}M) from the first one (4.75 x 10^{-6}M) we calculated the amount of Mn^{2+} originating from the inactive Mn^{2+}-protein complex. A value of 4 x 10^{-6}M was found. From the amount of protein (144 µg) and the mole-

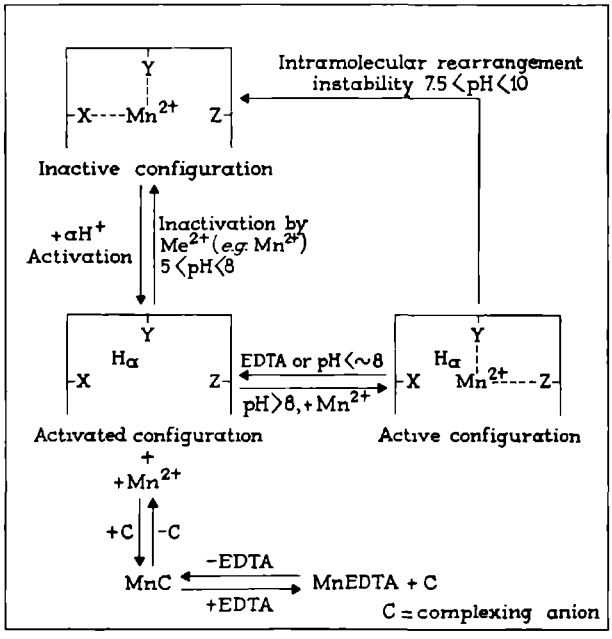
cular weight of 67 000 (VOGELS, 1966) the concentration of enzyme molecules was calculated; this yielded a value of 2.15 x 10^{-6}M. The ratio of moles of Mn^{2+} *vs.* moles of protein was 4 x 10^{-6} *vs.* 2.15 x 10^{-6} = 1.86 *vs.* 1.

7.8 DISCUSSION AND CONCLUSIONS

A decrease of the specific activity of allantoate amidohydrolase could be achieved 1) by the addition of bivalent cations, especially Mn^{2+}, at defined pH values and 2) by dilution of the enzyme with EDTA-free buffer. The first process was called inactivation, the second one instability. A comparison of these two effects was made (7.5). The arguments given there and the observation that enzyme, lowered in activity by either way, could be reactivated in a similar manner suggested that both phenomena were based on a similar mechanism, *viz.* inactivation by binding of bivalent cations to a defined site of the enzyme molecule. Inactivation only took place above pH 5. Then, the binding between Mn^{2+} and a particular enzyme ligand was strong enough to compete with protons for the same ligand. Therefore, it seemed likely that at least one carboxyl group was involved in binding. The strongest inactivation of the enzyme occurred at a pH of about 6. At pH values above 8 no inactivation by Mn^{2+} took place and Mn^{2+} protected the enzyme against the decrease of activity which occurred in the absence of this ion. Complexing agents (EDTA) exerted a similar effect: the EDTA concentration necessary to stabilize the enzyme was different at different pH values.

Both EDTA and Mn^{2+} were able to stabilize the enzyme above pH 8; below pH 8 only EDTA was able to protect the enzyme against a decrease of activity and Mn^{2+} inactivated the enzyme. The stabilizing effect of EDTA was probably due to its chelating capacity (7.5). Stabilization by Mn^{2+} ions above pH 8 was thought to be a prevention of an intramolecular rearrangement leading to inactivation (7.5).

The processes of activation, inactivation and instability and the reversibility of these processes could be represented by the following reaction sequence (Scheme 6):



Scheme 6

Schematic representation of the phenomena of activation, inactivation and instability of allantoate amidohydrolase from *Streptococcus allantoicus*. X, Y and Z represent three aminoacid residues in the active center of the enzyme.

Activation consists in a removal of Mn^{2+} from the inactive configuration of the enzyme. This displacement is at first brought about by protons and the free Mn^{2+} ions are subsequently bound to complexing substances, *viz.* buffer anions and/or EDTA (6.7). The activated configuration is only active above pH 8 in the presence of Mn^{2+} ions (5.5). At pH values below 8 the active configuration is converted to the inactive one. Mn^{2+} accelerates this conversion (7.2). EDTA may protect against this inactivation by binding of Mn^{2+} ions released from the active configuration. Above pH 8 Mn^{2+} ions are not strongly bound to the enzyme, since EDTA can inhibit the enzymic activity tested at optimal pH (5.5). Addition of a sufficient amount of Mn^{2+} restores the enzymic activity. Therefore, EDTA can convert the active configuration to the activated one above pH 8 and binds the Mn^{2+} ions which are released during this conversion. Thereby EDTA prevents an intra-

molecular rearrangement to the inactive configuration. Mn^{2+} ions exert a similar effect: presumably Mn^{2+} ions stabilize the enzyme above pH 8 by shielding one or more groups in the active center, *e.g.* X, and thus prevent the intramolecular rearrangement. This inactivation as a result of an intramolecular rearrangement, *viz.* instability, is abolished by subjecting the enzyme to an activation procedure.

This hypothesis is in accordance with the effects observed. Activation and inactivation are reversible processes and instability below pH 8 is due to inactivation by cations. From Fig.43 it already followed that at pH 8 the enzyme behaved exceptionally, *viz.* the instability of active enzyme was higher at pH 8 than at pH 7.5 and 8.5. We ascribe this to two different effects: 1) an inactivation by cations at pH values below 8 and 2) an instability of the enzyme above pH 8 as a result of an intramolecular rearrangement.

According to the classification used (6.3.1) allantoate amidohydrolase is a metal-enzyme complex. We consider this classification not fully adequate for the metal effects observed with this enzyme. At present only one manganese metalloenzyme is known (SCRUTTON *et al.*, 1966; MILDVAN *et al.*, 1966), *viz.* pyruvate carboxylase (pyruvate: carbon-dioxide ligase (ADP), EC 6.4.1.1).

An inactivation phenomenon somewhat similar to that of allantoate amidohydrolase was described for citrate lyase (citrate oxaloacetate-lyase, EC 4.1.3.6) by EISENTHAL, TATE and DATTA (1966). This enzyme lost activity rapidly in dilute solutions and the presence of Mg^{2+} largely prevented this instability. Protection against instability was dependent on the Mg^{2+} ion concentration. Furthermore, a partial reactivation was observed when Mg^{2+} ions were added after the activity had decreased by dilution (BLAIR, DATTA and TATE, 1967). The suggestion was made that the active enzyme was a Mg^{2+}-enzyme complex. Ca^{2+} also exerted a stabilizing effect but did not form a catalytically active complex with the enzyme. Presumably, a complex between metal ion and enzyme was formed at a site distinct from the active site. This would mean that a cofactor and a stabilizer bind to different sites of the enzyme.

The same was stated for carnosinase (aminoacyl-L-histidine hydrolase, EC 3.4.3.3) by ROSENBERG (1960a). In the absence of Mn^{2+} ions

the enzyme was unstable and reactivation could take place by addition of Mn^{2+}, Ca^{2+}, Mg^{2+}, Cd^{2+} and Zn^{2+}. The three cofactors (Mn^{2+}, Cd^{2+}, Zn^{2+}) displayed not the same stabilizing power and cofactor activity, *e.g.* Cd^{2+}, the best cofactor, showed less stabilizing power than Mn^{2+} (ROSENBERG, 1960b). Mg^{2+} and Ca^{2+}, which were no cofactors, were stabilizers. Two different sites on the enzyme were postulated to be available: one for stimulation, the other for stabilization.

The results obtained with citrate lyase and carnosinase did show that the activity of some enzymes decreased as a result of instability. The activity could be restored by the addition of several metal ions. This reaction process could be performed at the same pH at which instability occurred. On the contrary, allantoate amidohydrolase had to be subjected to an activation procedure to regain enzymic activity after decrease of activity by instability had occurred. This activation procedure consisted in a treatment of the enzyme at a defined pH in the presence of complexing substances.

In the case of allantoate amidohydrolase too, some metal ions which stabilized the enzyme had no cofactor activity, *e.g.* Hg^{2+}. Furthermore, not all cofactors, *e.g.* Mg^{2+}, exerted a stabilizing effect. The efficiency of cofactor activity was given by $Mn^{2+} > Mg^{2+} > Cd^{2+} > Co^{2+} > Ca^{2+} > Pb^{2+}$ (5.5) and that of stabilization at pH 8.5 by $Mn^{2+} > Hg^{2+} > Cd^{2+} > Pb^{2+} > Co^{2+} > Ni^{2+}$.

Stabilization of enzymes by EDTA might involve a totally different mechanism as stabilization by metal ions. A conformational change of enzyme structure in the presence of EDTA was proposed for fructose 1,6-diphosphatase from *Candida utilis* (ROSEN, ROSEN and HORECKER, 1965). At pH 7.5 no enzymic activity was obtained in the absence of EDTA, while EDTA had no effect at pH 9.5. Exposure of the enzyme to deionizing procedures failed to alter the EDTA requirement at pH 7.5 and therefore it would appear that the effect was not due to a removal of inhibitory metal ions from the enzyme. The authors assumed that EDTA may act direct on the enzyme altering its activity or that it may facilitate the binding of metal ions, necessary as cofactors, or substrate or both. However, no evidence was found in favor of such an explanation of the effect of complexing agents, like EDTA, on allantoate amidohydrolase.

Another enzyme which behaved unstable in solution was adenylate pyrophosphorylase (adenosine monophosphate: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) from tumor cells. Stabilization occurred in the presence of sulfate ions (VON HIPPEL and WONG, 1964). Allantoate amidohydrolase could not be stabilized by $(NH_4)_2SO_4$.

There are several more examples of enzymes which are 'inactivated' by dilution. In most cases this phenomenon was not understood, except when dissociation or aggregation took place. These effects will not be discussed here, since no evidence was obtained that such effects played a role in the inactivation of allantoate amidohydrolase.

A most interesting enzyme was L-aminoacid oxidase (L-aminoacid: oxygen oxidoreductase (deaminating), EC 1.4.3.2), which could be inactivated by incubation at 38°. This inactivation was time-, temperature- and pH-dependent and the equilibrium between active and inactive molecules was determined by temperature, pH and the amount of anions present during inactivation. Monovalent anions protected the enzyme against this decrease of activity, whereas bi- and trivalent anions accelerated it. Reactivation was obtained by treatment at pH 5 or by an excess of KCl (KEARNEY and SINGER, 1951a, b). Optical rotatory dispersion measurements revealed that a conformational change in the environment of the coenzyme FAD took place during the inactivation of the enzyme. By lowering the pH this change in structure was abolished, resulting in a reactivation (WELLNER, 1966).

MAGAR (1965) reported a conformational change on dilution of glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) but activity measurements were not performed.

From the above-mentioned considerations it is clear that none of the enzymes described behaved similar to allantoate amidohydrolase. A tentative explanation of inactivation and stabilization of this enzyme can be given (Scheme 6). This scheme fits well with the experimental results. More definite proof of the validity could probably result from some physical measurements.

The question arose whether we deal with a fortuitous property inherent to all known allantoate amidohydrolases, a hitherto unknown property of enzymes or the possibility that the inactive configuration of allantoate amidohydrolase is the active one of another enzyme.

SUMMARY

This thesis deals with the results of an experimental study of the metabolism of allantoin in *Streptococcus allantoicus* and especially with the elucidation of the phenomena of activation, inactivation and instability of the enzyme allantoate amidohydrolase.

In Chapter 1 the occurrence of allantoin and its metabolism is reviewed. Furthermore the effect of bivalent cations on some of these enzymes is mentioned and a review is given of metal-enzyme interactions.

Chapter 2 deals with the materials and methods used in the experimental work. Furthermore definitions are given which are used throughout this study.

In Chapter 3 the effects of bivalent cations and reducing substances on allantoinases from plants, bacterial and animal sources are investigated. These effects make it possible to classify four groups of allantoinases, each possessing another active configuration (Scheme 3).

In Chapter 4 the hydrolysis of allantoate in *Streptococcus allantoicus* is studied. The enzyme responsible for this degradation is allantoate amidohydrolase. This enzyme differs distinctly from allantoicase, which is found in microorganisms which grow under aerobic conditions on allantoin. The intermediate position of ureidoglycine is demonstrated from the production of ammonia, carbon dioxide, ureidoglycolate, glyoxylate and glycine during allantoate hydrolysis by purified allantoate amidohydrolase preparations. Furthermore, the non-enzymic decomposition of 5-aminohydantoin and its end products also indicate ureidoglycine being an intermediate. (-)-Ureidoglycolate formed during allantoate hydrolysis is converted by ureidoglycolase to glyoxylate and urea. Several non-enzymic reactions occur at the same time. The reactions catalyzed by allantoate amidohydrolase are summarized in Scheme 5.

In Chapter 5 some properties of allantoate amidohydrolase from *Streptococcus allantoicus* are described, *viz.* pH optimum, K_m, V_{max}, cofactors, inhibitors and heat-stability. The enzyme degrades allantoate, 4-methylallantoate and N-carbamoyl-L-asparagine. The latter degradation enables us to denote the bond attacked in allantoate and furthermore yields the absolute configuration of (-)-ureidoglycolate formed during allantoate hydrolysis, *viz.* (-)-ureidoglycolate = L-ureidoglycolate.

Chapter 6 deals with the phenomenon of activation of allantoate amidohydrolase from *Streptococcus allantoicus*. It is possible to activate the enzyme by choosing suitable activation conditions (pH, temperature, time of pretreatment and complexing ability of the activation mixture) at pH values between 1 and about 9. Activation consists in a removal of Mn^{2+} ions from the enzyme. These ions are incorrectly bound in the active center. The number of protons involved in this displacement reaction is determined from studies on the plateau and the initial rate of activation.

In Chapter 7 the phenomena of inactivation and stabilization are described. Inactivation by cations takes place between pH 5 and 8. The enzyme is unstable on dilution; stabilization occurs by EDTA and above pH 8 also by Mn^{2+} and some other bivalent cations. Decreases of enzymic activity as a result of inactivation or instability can be reversed by performing an activation step.

Inactivation by cations and the decrease of activity as a result of instability are thought to be based upon a similar mechanism, *viz.* binding of cations to ligands in the active center, resulting in an inactive enzyme. A special function is ascribed to Mn^{2+} ions. A hypothetical explanation of the phenomena of activation, inactivation and instability is given (Scheme 6).

SAMENVATTING

Dit proefschrift beschrijft een experimenteel onderzoek over het metabolisme van allantoïne in *Streptococcus allantoicus*. Deze bacterie groeit alleen onder anaerobe omstandigheden op allantoïne. Vooral het enzym allantoaat amidohydrolase, verantwoordelijk voor de allantoïnezuur afbraak in dit micro-organisme, werd uitvoerig bestudeerd.

In Hoofdstuk 1 wordt een kort overzicht gegeven van het voorkomen van allantoïne in de natuur en van zijn metabolisme (Schema's 1 en 2). Omdat de activiteit van veel enzymen betrokken bij het metabolisme van allantoïne beïnvloed wordt door bivalente kationen, wordt nader ingegaan op metaal-enzym wisselwerkingen in het algemeen.

Hoofdstuk 2 behandelt de in dit onderzoek toegepaste methodieken. Verder worden enkele definities gegeven, die tijdens het onderzoek gebruikt worden.

Het effect van bivalente kationen en reducerende stoffen op de activiteit van allantoïnases van verschillende oorsprong wordt in Hoofdstuk 3 behandeld.

Op grond van deze effecten is het mogelijk vier groepen van allantoïnases te onderscheiden (Schema 3).

De bestudering van de hydrolyse van allantoïnezuur door allantoaat amidohydrolase van *Streptococcus allantoicus* wordt in Hoofdstuk 4 beschreven. Dit enzym is niet identiek met het allantoïcase, het allantoïnezuur-hydrolyserend enzym, dat gevonden wordt in micro-organismen, die onder aerobe omstandigheden op allantoïne groeien. Uit de vorming van ammoniak, kooldioxide, ureïdoglycolzuur, glyoxylzuur en glycine tijdens de afbraak van allantoïnezuur door gezuiverde enzympreparaten blijkt, dat ureïdoglycine een tussenprodukt is. Dit wordt verder bevestigd door de resultaten verkregen bij de bestudering van de niet-enzymatische ontleding van 5-aminohydantoïne. Het enzym ureïdoglycolase hydrolyseert het, tijdens de afbraak van allantoïnezuur

gevormde, (-)-ureïdoglycolzuur tot ureum en glyoxylzuur. De enzymatische en niet-enzymatische reacties die optreden tijdens de afbraak van allantoïnezuur worden in Schema 5 weergegeven.

In Hoofdstuk 5 worden enkele eigenschappen van het allantoaat amidohydrolase van *Streptococcus allantoicus* besproken, zoals pH optimum, K_m, V_{max} en hitte-stabiliteit. Cofactoren en remmers van het enzym worden vermeld. Naast allantoïnezuur worden ook 4-methylallantoïnezuur en N-carbamoyl-L-asparagine gehydrolyseerd. De afbraak van de laatste stof maakt het mogelijk om sterische specificiteit van het enzym nader te bestuderen. De absolute configuratie van het gevormde (-)-ureïdoglycolzuur komt overeen met de L-configuratie.

Het activeringsproces van allantoaat amidohydrolase wordt in Hoofdstuk 6 besproken. Het enzym kan in de katalytisch actieve configuratie gebracht worden door een voorbehandeling onder geschikte omstandigheden (pH, temperatuur, tijd en complexerend vermogen tijdens voorbehandeling). Activering treedt op in het pH gebied tussen 1 en 9 en wordt als het afsplitsen van Mn^{2+} van het inactieve enzym beschouwd. Dit ion is gebonden aan 'verkeerde' liganden in het actieve centrum van het enzym. Het aantal protonen, betrokken bij de afsplitsing van Mn^{2+}, wordt berekend uit metingen van het evenwicht tussen actieve en inactieve enzym-moleculen en uit bepaling van de beginsnelheid van de activering.

De verschijnselen van inactivering en stabiliteit worden in Hoofdstuk 7 beschreven. Kationen inactiveren het actieve enzym tussen pH 5 en 8. Door verdunnen van de enzym-oplossing wordt het enzym instabiel; stabilisering wordt verkregen door EDTA en boven pH 8 ook door Mn^{2+} of enkele andere bivalente kationen toe te voegen. De afname van de enzymatische activiteit ten gevolge van inactivering of instabiliteit verdwijnt, wanneer het enzym wordt onderworpen aan een activeringsprocedure. Inactivering en instabiliteit worden beschouwd te berusten op hetzelfde mechanisme, te weten, het binden van kationen in het actieve centrum resulterend in een inactief enzym. Een hypothese, die de verschijnselen van activering, inactivering en instabiliteit verklaart, wordt in Schema 6 gegeven.

REFERENCES

ADACHI, K., Y.IWAYAMA, H.TANIOKA, and Y.TAKEDA. 1966. Purification and properties of homogentisate oxygenase from *Pseudomonas fluorescens*. Biochim.Biophys. Acta, 118: 88.

ALBERTY, R. A. 1956. Enzyme kinetics, p.1. In F.F.Nord (ed.), Advances Enzymol., Vol.17, Interscience Publ., New York.

ALEXANDER, B., G.LANDWEHR, and A.M.SELIGMAN. 1945. A specific micromethod for the colorimetric determination of glycine in blood and urine. J.Biol. Chem., 160: 51.

AMMANN, E.C.B., and V.H.LYNCH. 1964. Purine metabolism of unicellar algae. II. Adenine, hypoxanthine, and xanthine degradation by *Chlorella pyrenoidosa*. Biochim. Biophys. Acta, 87: 370.

AVIS, P.G., F.BERGEL, and R.C.BRAY. 1956. Cellular constituents. The chemistry of xanthine oxidase. Part III. Estimations of the co-factors and the catalytic activities of enzyme fractions from cow's milk. J.Chem.Soc., Part I, 1219.

BACH, S.J., and D.B.WHITEHOUSE. 1954. Purification and properties of arginase. Biochem. J., 57: xxxi.

BACHRACH, U. 1957. The aerobic breakdown of uric acid by certain Pseudomonads. J.Gen.Microbiol., 17: 1.

BARKER, H.A. 1961. Fermentations of nitrogenous organic compounds, p. 151. In I.C. Gunsalus and R.Y. Stanier (ed.), The Bacteria. A treatise on structure and function, Vol.II, Academic Press, New York.

BARTLEY, W., B.M.NORTON, and W.C.WERKHEISER. 1957. Determination of manganese in tissue extracts by means of formaldoxime. Biochem. J., 67: 291.

BEREZIN, I.V., G.Ya.KOLOMIITSEVA, A.V.LEVASKOV, and K.MARTINEK. 1966. Inhibitory effect of borates on the three-stage hydrolysis of nonspecific substrates by α-chymotrypsin. Dokl.Akad.Nauk SSSR, 171: 1213.

BLAIR, J.McD., S.P.DATTA, and S.S.TATE. 1967. Reversible inactivation of citrate lyase: effect of metal ions and adenine nucleotides. European J.Biochem., 1: 26.

BRINTZINGER, H. 1965. Metallionenkatalyse polarer Reaktionen. Z.Naturw.-Med. Grundlagenf., 2: 188.

BRODSKY, W.A., N.J.CARLISKY, C.F.GONZALES, and Y.E.SHAMOO. 1965. Metabolic pathways for urea production by the amphibian kidney. Am.J.Physiol., 208: 546.

BRUNEL, A. 1936. Le métabolisme de l'azote d'origine purique chez les champignons. Thèse Doct. Sci., Paris.

CASOLA, L., A.GIUDITTA, and E.ROCCA. 1964. Barbiturate inhibition of D-aspartate oxidoreductase: mechanism of action and structural requirements. Arch. Biochem. Biophys., 107: 57.

CHAN, P.C. 1967. Reversible effect of sodium dodecyl sulfate on human erythrocyte membrane adenosine triphosphatase. Biochim. Biophys. Acta, 135: 53.

CHOI, K.S., and A.H.ROUSH. 1965. Assay, purification and properties of glyoxylurease and allantoicase from the yeast *Candida utilis*. Federation Proc., 24: 594.

CHRISTENSEN, H.N., T.R.RIGGS, and N.E.RAY. 1951. Glycine determination in tissues. Anal. Chem., 23: 1521.

COHN, M. 1963. Magnetic resonance studies of metal activation of enzymic reactions of nucleotides and other phosphate substrates. Biochemistry, 2: 623.

COHN, M., and J.S.LEIGH. 1962. Magnetic resonance investigations of ternary complexes of enzyme-metal-substrate. Nature, 193: 1037.

COHN, M., and J.TOWNSEND. 1954. A study of manganous complexes by paramagnetic resonance absorption. Nature, 173: 1090.

COLE, S.W. 1904. Contributions to our knowledge on the action of enzymes. Part I. The influence of electrolytes on the action of amylolytic ferments. J.Physiol., 30: 202.

COLEMAN, D.L. 1966. Purification and properties of δ-aminolevulinate dehydratase from tissues of two strains of mice. J.Biol.Chem., 241: 5511.

COLEMAN, J.E., and B.L.VALLEE. 1961. Metallocarboxypeptidases: stability constants and enzymatic characteristics. J.Biol.Chem., 236: 2244.

DIXON, M., and E.C.WEBB. 1964. Enzymes, 2nd edition, p.523. Longmans, Green and Co., London.

DOMNAS, A. 1962. Amide metabolism in yeasts. II. The uptake of amide and amide like compounds by yeast. J.Biochem. (Tokyo), 52: 149.

EIGEN, M., and G.G.HAMMES. 1963. Elementary steps in enzyme reactions (as studied by relaxation spectrometry), p.1. In F.F.Nord (ed.), Advances Enzymol., Vol.25, Interscience Publ., New York.

EISENTHAL, R., S.S.TATE, and S.P.DATTA. 1966. Inactivation of citrate lyase by oxaloacetate and its structural analogues. Biochim.Biophys. Acta, 128: 155.

FLEMING, L.W., and G.W.CROSBIE. 1960. Non-enzymic transamination between glycine and glyoxylate. Biochim. Biophys. Acta, 43: 139.

FOSSE, R., et A.BRUNEL. 1939. Sur le ferment producteur d'acide allantoïque par hydratation d'allantoïne. Sa présence dans le règne animal. Compt.Rend., 188: 1067.

FRANKE, W. 1966. Allantoinase und Allantoicase, p.368. In Hoppe-Seyler/Thierfelder, Handbuch der physiologisch- und pathologisch-chemischen Analyse, 10. Aufl., Bd. VI/C.

FRANKE, W., A.THIEMANN, C.REMILY, L.MÖCHEL, und K.HEYE. 1965. Zur Kenntnis ureidspaltender Enzyme. I. Soja-Allantoinase. Enzymologia, 29: 251.

GINSBURG, A., and A.H.MEHLER. 1966. Specific anion binding to fructose diphosphate aldolase from rabbit muscle. Biochemistry, 5: 2623.

GOORE, M.Y., and J.F.THOMSON. 1967. γ-Glutamyl transpeptidase of kidney bean fruit. II. Studies on the activating effect of sodium citrate. Biochim.Biophys. Acta, 132: 27.

GOPINATHAN, K.P., M.SIRSI, and C.S.VAIDYANATHAN. 1964. Nicotinamide-adenine dinucleotide glycohydrolase of *Mycobacterium tuberculosis* $H_{37}R_v$. Biochem. J., 91: 277.

GUITTON, Y., G.DURAND, et A.BRUNEL. 1965. Le métabolisme de l'azote chez les microorganismes et les végétaux supérieurs. I. L'urée.- II. Les uréides. Bull.Soc.Physiol.Végét., 11: 3.

GURD, F.R.N., and P.E.WILCOX. 1956. Complex formation between metallic cations and proteins, peptides, and amino acids, p.311. In M.L.Anson and J.T. Edsall (ed.), Advances Protein Chem., Vol.11, Academic Press, New York.

HAGUE, D.N., and M.EIGEN. 1966. Role of metals in enzymatic reactions. Part I. Kinetics of complex formation in model systems involving magnesium and 8-hydroxyquinoline. Trans.Faraday Soc., 62: 1236.

HEYNEMAN, R.A., and R.E.VERCAUTEREN. 1964. Activation of the latent phenol-oxidase of *Tenebrio molitor*. Enzymologia, 28: 85.

HOFSTEE, B.H.J. 1952. On the evaluation of the constants V_M and K_M in enzyme reactions. Science, 116: 329.

HOFSTEE, B.H.J. 1959. Non-inverted versus inverted plots in enzyme kinetics. Nature, 184: 1296.

HORI, M., and J.F.HENDERSON. 1966. Purification and properties of adenylate pyrophosphorylase from Ehrlich ascites tumor cells. J.Biol.Chem., 241: 1406.

IRVING, H., and R.J.P.WILLIAMS. 1953. The stability of transition-metal complexes. J.Chem.Soc., Part III, 3192.

KÄGI, J.H.R., and B.L.VALLEE. 1960. The role of zinc in alcohol dehydrogenase. V. The effect of metal-binding agents on the structure of the yeast alcohol dehydrogenase molecule. J.Biol.Chem., 235: 3188.

KEARNEY, E.B., and T.P.SINGER. 1951a. The L-amino acid oxidases of snake venom. III. Reversible inactivation of L-amino acid oxidases. Arch. Biochem. Biophys., 33: 377.

KEARNEY, E.B., and T.P.SINGER. 1951b. The L-amino acid oxidases of snake venom. IV. The effect of anions on the reversible inactivation. Arch.Biochem. Biophys., 33: 397.

KENTEN, R.H. 1957. Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. 1. Activation by acid and alkali. Biochem. J., 67: 300.

KREBS, E.G., and E.H.FISCHER. 1956. The phosphorylase *b* to *a* converting enzyme of rabbit skeletal muscle. Biochim.Biophys.Acta, 20: 150.

LARSSON-RAŹNIKIEWICZ, M. 1964. Kinetic studies on the reaction catalyzed by phosphoglycerate kinase. I. The effect of Mg^{2+} and adenosine 5'-triphosphate. Biochim. Biophys. Acta, 85: 60.

LARSSON-RAŹNIKIEWICZ, M. 1967. Kinetic studies on the reaction catalyzed by phosphoglycerate kinase. II. The kinetic relationships between 3-phosphoglycerate, $MgATP^{2-}$ and activating metal ion. Biochim. Biophys. Acta, 132· 33.

LARSSON-RAŹNIKIEWICZ, M., and B.G.MALMSTRÒM. 1961. The metal ion activation of 3-phosphoglycerate kinase in correlation with metal-binding studies. Arch. Biochem. Biophys., 92: 94.

LASKOWSKI, M. 1951. Allantoinase and allantoicase, p.946. In J.B.Sumner and K.Myrback (ed.), The Enzymes. Chemistry and mechanism of action, Vol. I, part 2, Academic Press, New York.

LEE, K.W., and A.H.ROUSH. 1964. Allantoinase assays and their application to yeast and soybean allantoinases. Arch. Biochem.Biophys., 108: 460.

LINDSKOG, S., and B.G.MALMSTRÖM. 1962. Metal binding and catalytic activity in bovine carbonic anhydrase. J.Biol.Chem., 237: 1129.

LOWRY, O.H., N.J.ROSEBROUGH, A.L.FARR, and R.J.RANDALL. 1951. Protein measurement with the Folin phenol reagent. J.Biol.Chem., 193· 265.

MAGAR, M.E. 1965. Conformational changes in glutamate dehydrogenase. Biochim. Biophys. Acta, 96· 345.

MALMSTRÒM, B.G. 1961. Role of metal-binding in enzymic reactions. Federation Proc., 20 60.

MALMSTRÖM, B.G., and A.ROSENBERG. 1959. Mechanism of metal ion activation of enzymes, p.131. In F.F.Nord (ed.), Advances Enzymol., Vol.21, Interscience Publ., New York.

MALMSTRÒM, B.G., T.VANNGÅRD, and M.LARSSON. 1958. An electron-spin-resonance study of the interaction of manganous ions with enolase and its substrate. Biochim. Biophys. Acta, 30· 1.

MANN, P.J.G., and B.WOOLF. 1930. Action of salts on fumarase. I. Biochem. J., 24· 427.

MASSEY, V. 1953. Studies on fumarase. 2. The effects of inorganic anions on fumarase activity. Biochem. J., 53. 67.

METZLER, D.E., J.OLIVARD, and E.E.SNELL. 1954. Transamination of pyridoxamine and amino acids with glyoxylic acid. J.Am.Chem.Soc., 76 644.

MILDVAN, A.S., and M.COHN. 1965. Kinetic and magnetic resonance studies of the pyruvate kinase reaction. I. Divalent metal complexes of pyruvate kinase. J.Biol.Chem., 240 238.

MILDVAN, A.S., and M.COHN. 1966. Kinetic and magnetic resonance studies of the pyruvate kinase reaction. II. Complexes of enzyme, metal and substrates. J. Biol.Chem., 241. 1178.

MILDVAN, A.S., M.C.SCRUTTON, and M.F.UTTER. 1966. Pyruvate carboxylase. VII. A possible role for tightly bound manganese. J.Biol.Chem., 241· 3488.

MORRISON, J.F., and M.L.UHR. 1966. The function of bivalent metal ions in the reaction catalyzed by ATP: creatine phosphotransferase. Biochim.Biophys. Acta, 122: 57.

MONOD, J., J.P.CHANGEUX, and F.JACOB. 1963. Allosteric proteins and cellular control systems. J.Mol.Biol., 6: 306.

MOTHES, K., und L.ENGELBRECHT. 1953. Über die Beziehungen des Glykolsäure-Glyoxylsäure Systems zum Allantoin. Z.Physiol.Chem., 295: 387.

MUUS, J., 1953. Studies on salivary amylase with special reference to the interaction with chloride ions. Compt. Rend. Trav. Lab. Carlsberg. Série Chimique, 28: 317.

MYRBÄCK, K. 1926. Über Verbindungen einiger Enzyme mit inaktivierenden Stoffen. II. Z.Physiol. Chem., 159: 1.

NAKADA, H.I., and S.WEINHOUSE. 1953. Non-enzymatic transamination with glyoxylic acid and various amino acids. J.Biol.Chem., 204: 831.

NÉMETHY, G. 1967. Hydrophobe Wechselwirkungen. Angew.Chem., 79: 260.

OGASAWARA, N., J.E.GANDER, and L.M.H.HENDERSON. 1966. Purification and properties of 3-hydroxyanthranilate oxygenase from beef kidney. J.Biol. Chem., 241: 613.

O'SULLIVAN, W.J., and M.COHN. 1966. Magnetic resonance investigations of the metal complexes formed in the manganese-activated creatine kinase reaction. J.Biol.Chem., 241: 3104.

PERSON, P., J.H.FELTON, and H.ZIPPER. 1965. Disruption of mitochondria and solubilization of cytochrome oxidase by NaOH and KOH. Biochim. Biophys. Acta, 105: 393.

RABIN, B.R., and E.M.CROOK. 1956. The activation of enzymes by metal ions. Biochim. Biophys. Acta, 19: 550.

RAGETLI, H.W.J., M.WEINTRAUB, and U.M.RINK. 1966. Latent acid phosphatase in chloroplasts. Can. J.Botany, 44: 1723.

RAO, N.A.N., and T.RAMAKRISHNAN. 1962. The interaction of glyoxylate with cysteine and its application to the assay of isocitritase and of transaminases involving glyoxylate. Biochim. Biophys. Acta, 58: 262.

RAY, W.J. Jr., and G.A.ROSCELLI. 1966. Activation and inhibition in the phosphoglucomutase system. J.Biol.Chem., 241: 2596.

ROSEN, O.M., S.M.ROSEN, and B.L.HORECKER. 1965. Purification and properties of a specific fructose 1,6-diphosphatase from *Candida utilis*. Arch.Biochem.Biophys., 112: 411.

ROSENBERG, A. 1960a. Studies on carnosinase. III. Metal-ion stabilization of the enzyme. Ark. Kemi, 17: 25.

ROSENBERG, A. 1960b. The activation of carnosinase by divalent metal ions. Biochim.Biophys. Acta, 45: 297.

RYDER, E., C.GREGOLIN, H.C.CHANG, and M.D.LANE. 1967. Liver acetyl CoA carboxylase: insight into the mechanism of activation by tricarboxylic acids

and acetyl CoA. Proc.Natl.Acad.Sci. U.S., 57: 1455.

SALAS, J., M.SALAS, E.VIÑUELA, and A.SOLS. 1965. Glucokinase of rabbit liver. Purification and properties. J.Biol.Chem., 240: 1014.

SCHERAGA, H.A. 1963. Intramolecular bonds in proteins. II. Non-covalent bonds, p. 477. In H.Neurath (ed.), The Proteins. Composition, structure and function, Vol. 1, Academic Press, New York.

SCHLESINGER, M.J. 1966. Activation of a mutationally altered form of the *Escherichia coli* alkaline phosphatase by zinc. J.Biol.Chem., 241: 3181.

SCRUTTON, M.C., M.F.UTTER, and A.S.MILDVAN. 1966. Pyruvate carboxylase. VI. The presence of tightly bound manganese. J.Biol.Chem., 241: 3480.

SMITH, E.L. 1951. The specificity of certain peptidases, p.191. In F.F. Nord (ed.), Advances Enzymol., Vol.12, Interscience Publ., New York.

STETTEN, M.R., and F.F.BURNETT. 1966. Activation of rat-liver microsomal glucose-6-phosphatase, inorganic pyrophosphatase and inorganic pyrophosphate-glucose phosphotransferase by hydroxyl ion. Biochim. Biophys. Acta, 128: 344.

STEWARD, F.C., and J.K.POLLARD. 1957. Nitrogen metabolism in plants: Allantoin and allantoic acid. Ten years in retrospect. Ann. Rev. Plant Physiol., 8: 88.

SUMNER, J.B. 1951. Urease, p.873. In J.B.Sumner and K.Myrbäck (ed.), The Enzymes. Chemistry and mechanism of action, Vol.I, part 2, Academic Press, New York.

SWARTZ, M.N., N.O.KAPLAN, and M.E.FRECH. 1956. Significance of 'heat-activated' enzymes. Science, 123: 50.

THEORELL, H. 1958. Kinetics and equilibrium in the liver alcohol dehydrogenase system, p. 31. In F.F.Nord (ed.), Advances Enzymol., Vol. 20, Interscience Publ., New York.

TRIJBELS, J.M.F. 1967. An enzymic study of the metabolism of allantoin in various organisms. Thesis, Nijmegen.

TRIJBELS, F., and G.D.VOGELS. 1966a. Degradation of allantoin by *Pseudomonas acidovorans*. Biochim.Biophys.Acta, 113: 292.

TRIJBELS, F., and G.D.VOGELS. 1966b. Allantoicase and ureidoglycolase in Pseudomonas and Penicillium species. Biochim.Biophys. Acta, 118: 387.

TRIJBELS, F., and G.D.VOGELS. 1967. Allantoate and ureidoglycolate degradation by *Pseudomonas aeruginosa*. Biochim.Biophys. Acta, 132: 115.

VAGELOS, P.R., A.W.ALBERTS, and D.B.MARTIN. 1962. Activation of acetyl-CoA carboxylase and associated alteration of sedimentation characteristics of the enzyme. Biochem.Biophys.Res.Commun., 8: 4.

VAGELOS, P.R., A.W.ALBERTS, and D.B.MARTIN. 1963. Studies on the mechanism of activation of acetyl coenzyme A carboxylase by citrate. J.Biol.Chem., 238: 533.

VALENTINE, R.C., R.BOJANOWSKI, E.GAUDY, and R.S.WOLFE. 1962. Mechanism

of the allantoin fermentation. J.Biol.Chem., 237 2271.

VALENTINE, R.C., and R.S.WOLFE. 1960. Phosphorolysis of carbamyl oxamic acid. Biochim. Biophys. Acta, 45 389.

VALENTINE, R.C., and R.S.WOLFE. 1961a. Glyoxylurea. Biochem.Biophys. Res. Commun., 5 305.

VALENTINE, R.C., and R.S.WOLFE. 1961b. Phosphate-dependent degradation of urea. Nature, 191 925.

VALLEE, B.L. 1955. Zinc and metalloenzymes, p.317. In M.L.Anson and J.T. Edsall (ed.), Advances Protein Chem., Vol. 10, Academic Press, New York.

VALLEE, B.L. 1960. Metal and enzyme interactions correlation of composition, function, and structure, p. 225. In P.D. Boyer, H. Lardy and K.Myrback (ed.), The Enzymes, Vol.3, part B, Academic Press, New York.

VALLEE, B.L., and J.E.COLEMAN. 1964. Metal coordination and enzyme action, p.165. In M.F. Florkin and E.H. Stotz (ed.), Comprehensive Biochemistry, Vol.12, Elsevier Publ. Co., Amsterdam.

VALLEF, B.L., E.A.STEIN, W.N.SUMERWELL, and E.H.FISCHER. 1959. Metal content of α-amylases of various origines. J.Biol.Chem., 234· 2901.

VAN DER DRIFT, C., and G.D.VOGELS. 1966. Allantoin and allantoate in higher plants. Acta Botan. Neerl., 15 209.

VAN DER DRIFT, C., and G.D.VOGELS. 1967. Activation and inactivation of allantoate amidohydrolase. Biochim.Biophys. Acta, 139 162.

VOGELS, G.D. 1960. Onderzoekingen over de afbraak van allantoine door Streptococcen. Jaarverslag van de Stichting Delfts Hogeschoolfonds 1959-1960: 43.

VOGELS, G.D. 1963. On the microbial metabolism of allantoin. Thesis, Delft.

VOGELS, G.D. 1966. Reversible activation of allantoate amidohydrolase by acid-pretreatment and other properties of the enzyme. Biochim. Biophys. Acta, 113: 277.

VOGELS, G.D., F.TRIJBELS, and A.UFFINK. 1966. Allantoinases from bacterial, plant and animal sources. I. Purification and enzymic properties. Biochim. Biophys. Acta, 122 482.

VOGELS, G.D., and C.VAN DER DRIFT. 1966. Allantoinases from bacterial, plant and animal sources. II. Effect of bivalent cations and reducing substances on the enzymic activity. Biochim. Biophys. Acta, 122· 497.

VON HIPPEL, P.H., and K.Y.WONG. 1964. Neutral salts: The generality of their effects on the stability of macromolecular conformations. Science, 145 577.

WALLACH, D.P., and S.GRISOLIA. 1956. The purification and properties of hydropyrimidine hydrase. J.Biol.Chem., 226 277.

WARREN, J.C., and S.G.CHEATUM. 1966. Effect of neutral salts on enzyme activity and structure. Biochemistry, 5: 1702.

WEBB, J.L. 1966. Enzyme and metabolic inhibitors, p.595. Vol.III, Academic Press, New York.

WEBB, E.C., and P.F.M.MORROW. 1959. The activation of an arylsulphatase from

ox liver by chloride and other anions. Biochem.J., 73: 7.

WEBSTER, L.T. Jr. 1967. Studies of the acetyl coenzyme A synthetase reaction. V. The requirement for monovalent and divalent cations in partial reactions involving enzyme-bound adenylate. J.Biol.Chem., 242: 1232.

WELLNER, D. 1966. Evidence for conformational changes in L-amino acid oxidase associated with reversible inactivation. Biochemistry, 5: 1585.

WILLIAMS, R.J.P. 1959. Coordination, chelation and catalysis, p.391. In P.D.Boyer, H. Lardy and K.Myrbäck (ed.), The Enzymes, Vol.1, Academic Press, New York.

WILLIAMS, R.J.P. 1961. The selective interaction of metal ions and protein groups, p.133. In Proc.V Intern.Congres Biochem., Vol. IV, Pergamon Press, Oxford.

ZITTLE, C.A. 1951. Reaction of borate with substances of biological interest, p. 493. In F.F.Nord (ed.), Advances Enzymol., Vol. 12, Interscience Publ., New York.

STELLINGEN

1

Het bepalen van de allantoïcase activiteit door directe koppeling van glyoxylzuur met phenylhydrazine verdient geen aanbeveling.

Lee, K.W. en A.H.Roush, Arch. Biochem. Biophys., 108 (1964) 460.

2

Het door Cook en Boulter beschreven metabolisme van ureum in *Candida flareri* is onvoldoende bewezen.

Cook, A.R. en D.Boulter, Phytochemistry, 3 (1964) 313.

3

De gewijzigde regel van Patterson-Brode voor het bepalen van de absolute configuratie van α-aminozuren is niet altijd toepasbaar.

Greenstein, J.P. en M.Winitz, Chemistry of the amino acids, Vol.1 (1961) 117.

4

De bepaling van de glomerulaire filtratiesnelheid met behulp van een vitamine B_{12} verdwijningscurve is onnauwkeurig.

Troelstra, J.A., Mndschr. Kindergeneesk., 35 (1967) 213.

5

De aanwezigheid van messenger activiteit in RNA geïsoleerd uit hersenkernen is onvoldoende bewezen door de metingen van Bondy en Roberts.

Bondy, S.C. en S.Roberts, Biochem. J., 105 (1967) 1110.

6

De door Kok en Varner voorgestelde methode voor opsporing van buitenaards leven is niet bevredigend.

Kok, B. en J.E.Varner, Science, 155 (1965) 1110.

7

De metingen van Brown laten geen conclusies toe omtrent de eigenschappen van (Na^+-K^+)-geactiveerde ATPase in konijnehart.

Brown, H.D., Biochim.Biophys. Acta, 120 (1966) 162.

8

Door de proeven van Trione c.s. is niet bewezen dat sporogene verbindingen uit schimmels absorberen bij een golflengte van 310 mμ.

Trione, E.J., C.M.Leach en J.T.Mutch, Nature 212 (1966) 163.

9

Bij het bepalen van histidine in eiwitten met behulp van diazonium-1H-tetrazole wordt onvoldoende rekening gehouden met de aanwezigheid van tyrosine.

Horinishi, H., Y.Hachimori, K.Kurihara en K.Shibata, Biochim. Biophys. Acta, 86 (1964) 477.

Bak, T.G. en R.Sato, Biochim. Biophys. Acta, 146 (1967) 328.

10

Het urinezuur gehalte in serum kan niet beschouwd worden als maat voor de intelligentie.